Utilising the therapeutic potential of xenin and related molecules for diabetes and obesity

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Philippians 4:13

SUMMARY

The diminished incretin effect is a pathological characteristic of type 2 diabetes mellitus (T2DM), caused by reduced secretion of glucagon-like-peptide-1 (GLP-1) and blunted biological actions of glucose-dependent insulinotropic polypeptide (GIP). Presently, clinically approved GLP-1 analogues, targeting the GLP-1 arm of the incretin axis, help to overcome the diminished GLP-1 secretion. However, currently there is no clinically approved therapeutic agent to potentiate the biological actions of GIP. Furthermore, established pharmacological agents currently fail to replicate the benefits of bariatric surgery, which along with increasing prevalence increases both health and socio-economic burdens. Likewise, due to the multifactorial characteristics, current monotherapies fail to elicit persistent effective management. Therefore, novel, more-effective treatment paradigms are required to address these unmet needs. The gut hormone xenin has been reported to potentiate biological actions of GIP, with truncated analogues of xenin displaying more potent antidiabetic effects. This thesis characterised the GIP-potentiating effects of xenin-based therapies, along with their therapeutic applicability for diabetes and/or obesity. This included xenin-8-Gln, as part of a hybrid agent- (DAla^2)GIP/xenin-8-Gln, multiple novel xenin hexapeptides, as well as TNP-470, an angiogenesis inhibitor shown to increase xenin concentrations. Importantly, in all therapies assessed, there was clear augmentation of GIP action, resulting in enhanced metabolic effects. (DAla^2)GIP/xenin-8-Gln administered in combination with exendin-4 demonstrated metabolic benefits that were persistent in nature, which is highly favourable in such a progressive disease. Further characterisation of truncated xenin peptides showed that ψ-xenin-6 was the most potent truncated hexapeptide assessed, with GIP-potentiating and satiety effects shown, along with biological effects evident up to 8 hours. Furthermore, repeated

administration of ψ-xenin-6 in combination with sitagliptin further augmented the efficacy of sitagliptin, with effects more prominent and rapid with combined modulation. Additionally, beneficial efficacy of sitagliptin was augmented through combined administration with TNP-470, displaying improvements in glucose tolerance, insulin sensitivity and significant anti-obesity effects. Together, these findings provide reinforcing evidence for development of novel xenin-based therapies, which act to potentiate biological actions of GIP, and the need for combinational therapeutic approaches to target multiple pathways to help alleviate hyperglycaemia in T2DM, with potential for translation to the clinic.

ABBREVIATIONS

AAC	Area above the curve
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AMC	7-amino-4-methylcoumarin
AMPK	AMP activated protein kinase
ATP	Adenosine triphosphate
AUC	Area under the curve
BMC	Bone mineral content
BMI	Body mass index
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic-AMP (3, 5'-cyclic monophosphate)
CCK	Cholecystokinin
CLAMS	Comprehensive Laboratory Animal Monitoring System
CPM	Counts per minute
Da	Dalton(s)
DEXA	Dual-energy X-ray absorptiometry
dH_2O	Distilled water
DPP-4	Dipeptidyl peptidase-4
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FFA	Free fatty acids
FGF21	Fibroblast growth factor 21
Fig.	Figure
g	Gram(s)
GIP	Glucose-dependent insulinotropic polypeptide
GIP-R	Glucose-dependent insulinotropic polypeptide receptor

GLP-1	Glucagon like peptide-1
GLP-1R	Glucagon like peptide-1 receptor
GLUT	Glucose transporter
G6Pase	Glucose 6-phosphatase
h	Hour(s)
HBSS	Hank’s balanced salt solution
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethyl-piperazine-N’-2-thane-sulphonic acid
HFF	High fat fed
HNF1A	Hepatocyte nuclear factor 1 homeobox A
HNF1B	Hepatocyte nuclear factor 1 homeobox B
HNF4A	Hepatocyte nuclear factor 4 alpha
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
IAA	Insulin autoantibodies
ICA	Islet cell autoantibodies
IDF	International Diabetes Federation
IgG	Immunoglobulin G
IL-6	Interleukin 6
IRS-1	Insulin receptor substrate-1
K^+	Potassium
KCl	Potassium chloride
Kg	Kilogram(s)
KRBB	Krebs ringer bicarbonate buffer
l	Litre(s)
M	Molar
m/z	Mass-to-charge ratio

MALDI-TOF MS	Matrix-Assisted Laser Desorption - Time of Flight Mass Spectrometry
METAP2	Methionine aminopeptidase 2
mg	Milligram
MHC	Major histocompatibility complex
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mmol	Millimole(s)
MODY	Maturity-onset diabetes of the young
n	Number of observations
NEFA	Non-esterified fatty acids
ng	Nanogram(s)
nM	Nanomolar
nmol	Nanomole(s)
Ntsr 1	Neurotensin receptor subtype 1
PAL	Palmitate
PBS	Phosphate buffered saline
PDX1	Pancreatic and duodenal homeobox-1
PEPCK	Phosphoenolpyruvate carboxykinase
PI	Phosphatidylinositol
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PP	Pancreatic polypeptide
PYY	Peptide tyrosine-tyrosine
RIA	Radioimmunoassay
RNA	Ribonucleic acid

rpm	Revolutions per minute
RT	Retention time
S.E.M.	Standard error of the mean
sec	Second(s)
SGLT-2	Sodium/glucose cotransporter 2
SNAC	(Sodium N-[8-(2-hydroxybenzoyl)Amino]Caprylate
STZ	Streptozotocin
t	Time
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TCA	Tricarboxylic acid cycle
TFA	Trifluoroacetic acid
TNF-α	Tumor Necrosis Factor Alpha
U	Unit(s)
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
µg	Microgram(s)
µl	Microlitre(s)

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PUBLICATIONS ARISING FROM THIS THESIS

Chapter 1:
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ABSTRACTS

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Chapter 1

General Introduction

1.1 DIABETES MELLITUS

Diabetes mellitus is a complex progressive metabolic disease that occurs due to pancreatic beta cell dysfunction and/or insulin resistance, and manifests itself through chronically elevated glucose concentrations. Consequently, patients with diabetes require continuous monitoring and control of their blood glucose levels mainly through exogenous drug therapy. According to recent statistics compiled by the International Diabetes Federation (2019), 463 million adults have diabetes, which is expected to rise to 700 million by 2045, with 4.2 million deaths attributed to diabetes and its associated complications each year [IDF, 2019]. It is estimated that by 2030 diabetes will be the seventh leading cause of mortality worldwide [WHO, 2011]. This metabolic disorder has become a global epidemic with major contributing factors including obesity, physical inactivity and an excessive calorie consumption. Diabetes has become a global economic burden, due to its ever-increasing healthcare expenditures, along with its escalating rates of mortality and morbidity and has become one of the biggest health care challenges of the 21st century [Lu *et al*. 2014]. Diabetes accounts for nearly 12% of global health expenditure and globally cost more than 760 billion dollars in 2019 [IDF, 2019], accounting for over 10% of the NHS budget in the UK. Therefore, these staggering statistics clearly highlight the challenge that diabetes presents, and the vital need to develop new and more efficacious treatments.

1.1.1 TYPES OF DIABETES

The two main forms of diabetes are; type 1 diabetes mellitus (T1DM), which accounts for 10% of all diagnosed cases, and type 2 diabetes mellitus (T2DM), which is the more prevalent type, accounting for 90% of all diagnosed cases. Clinically, both types

share some similarities, including presenting indications such as polydipsia or polyuria. However, due to their variances in aetiology, they are considered to be at opposite ends of a spectrum in relation to insulin production and function.

In T1DM, patients are dependent on insulin due to the irreversible, cell-mediated destruction of the pancreatic beta cells, resulting in complete insulin deficiency. Therefore, the disturbance of insulin production in T1DM is the main feature of this type. T1DM usually has a juvenile onset and is associated with genetic susceptibility inherited via the major histocompatibility complex (MHC) (HLA class II region on chromosome 6p21) genes encoding DQ and DR [Nobel and Valdes, 2011]. The autoimmune response in susceptible type 1 individuals can be triggered by environmental factors [Knip and Simell, 2012], such as toxins or foods, resulting in the abnormal stimulation of B-cell and T-cell mediated immune response. This initiates the production of autoantibodies, including islet cell autoantibodies (ICA), insulin autoantibodies (IAA), autoantibodies to glutamic acid decarboxylase (GADA) and autoantibodies to tyrosine phosphatases (IA-2a and IA-2b) [Sørgjerd, 2019] against beta cell antigens and triggers insulitis, with subsequent destruction of the pancreatic insulin producing beta cells, creating absolute insulin deficiency.

However, unlike T1DM, patients with T2DM have preserved ability to produce insulin from their beta cells, but due to insulin resistance, peripheral cells fail to respond to insulin. Therefore, it is the disturbance of insulin action from the diminished response of peripheral tissues to insulin in T2DM that is the main feature [Ta, 2014]. Albeit, T2DM patients also present with decreased beta cell mass. This means that the remaining beta cells have an increased workload, leading to further decline and failure

of the beta cells, resulting in subsequent loss of insulin secretion. There are various factors that contribute to development of T2DM, and these include poor lifestyle choices and obesity, as well as genetic factors linked to beta cell dysfunction [Colberg, 2010]. The diminishing effects of insulin secretion and action in T2DM are related to increased levels of ectopic fat storage, increased secretion of adipokines and elevated non-esterified fatty acids in circulation. T2DM was previously more commonly diagnosed in adulthood, with predisposition linked with ethnicity and family history [Oldroyd *et al*. 2005]. However, age onset for this disorder has decreased, with incidence of T2DM increasing in children. This reflects the rise in childhood obesity [Rosenbloom, 2002], along with other factors, including lack of physical activity and diets high in saturated fats.

Other types of diabetes include gestational diabetes, which affects approximately 3-5% of all pregnancies and develops between the 24^{th} and 28^{th} week of pregnancy. The babies born from mothers with gestational diabetes and the women themselves have an increased risk of developing T2DM later in life. Maturity-onset diabetes of the young (MODY) is another type of diabetes, which usually develops before the age of 25 affecting around 1-2% of people with diabetes. There are now at least 14 known MODY-causing mutations, which vary dependent on age onset and treatment response [Hoffman and Jialal, 2019]. The four most common mutations are HNF1B, GCK, HNF1A and HNF4A, however other mutations include INS, NEURO1, PDX1, PAX4, ABCC8, KCNJ11, KLF11, CEL, BLK and APPL1 [Hoffman and Jialal, 2019].

1.1.2 ISLETS OF LANGERHANS

Islets of Langerhans were first identified as clusters of endocrine cells scattered throughout the pancreas by Paul Langerhans in 1869. The normal human pancreas contains about 1,000,000 islets, which play critical roles in energy homeostasis, nutrient metabolism and controlling blood glucose levels through the secretion of insulin. These islets consist of five different cell types including alpha cells, beta cells, delta cells, pancreatic polypeptide-producing (PP) cells and epsilon cells [Scharfmann *et al*. 2008]. The alpha and beta cells are responsible for secretion of glucagon and insulin respectively, and are the most abundant, playing vital roles in the maintenance of glucose homeostasis [Bosco, 2010]. Somatostatin secreted by pancreatic delta cells, inhibits secretion of insulin and glucagon [Briant *et al*. 2016]. The PP cells, which express pancreatic polypeptide, have an inhibitory response on pancreatic exocrine secretion [Kojima *et al*. 2007]. The epsilon cells, which secrete ghrelin, comprise less than 1% of pancreatic cells. It has been suggested that ghrelin has roles in suppressing insulin secretion and it possesses hyperglycaemic effects [Broglio *et al*. 2001]. The structure of islets are highly species specific. In rodent islets, beta cells encompass 70-80% of the pancreatic cells and are localised at the core. In contrast, alpha-, delta- and PP cells are located at the periphery of the islet, known classically as the islet mantle [Cabrera *et al*. 2006; Steiner *et al*. 2010]. Unlike the defined beta cell core and alpha cell periphery of rodent islets, human islets maintain a more scattered endocrine cell organisation [Steiner *et al*. 2010; Cabrera *et al*. 2006]. Islets have a rich blood and nervous supply, with vascularisation and innervation playing key roles in islet function [Diez *et al.* 2017]. In the various aetiologies of diabetes aforementioned, the unique architectural organisation and interaction between these different islet cell types is

disturbed, therefore resulting in dysfunction of the islet cells [Rahier *et al*. 1983; Brereton *et al*. 2015].

1.1.3. INSULIN

Insulin, a 51-amino acid peptide with a molecular weight of 5805 Da [Mangesh *et al*. 2014], is synthesised within the ribosomes of the endoplasmic reticulum (ER) [Vargas and Sepulveda, 2018]. Insulin is initially synthesised as a single-chain 110-amino acid precursor polypeptide, preproinsulin, which is cleaved forming proinsulin [Patzelt *et al*. 1978], and transported to the Golgi apparatus where it is further cleaved by multiple specific endopeptidases, generating the mature form of insulin and C-peptide. Insulin is subsequently packed into secretory vesicles within beta cells, prior to the stimulation of insulin release by exocytosis [Shpakov, 2015].

The secretion of insulin is dependent on the ability of beta cells to respond appropriately to physiological stimuli, predominately changes in plasma glucose concentrations, by turning increased glycolytic metabolism into exocytotic release of insulin [Squires *et al*. 2005]. The occurrence of insulin secretion is biphasic, involving triggering (first phase) and amplification (second phase) pathways. These insulin granules comprised in beta cells can either be readily available for release; first phase of insulin release or present as reserve pools; second phase of insulin release, which involves transformation from reserve to readily releasable before exocytosis [Bratanova-Tochkova *et al*. 2002]. Pancreatic beta cells express a high volume of GLUT2 transporters, but human beta cells express predominantly GLUT1 transporters, which ensure that any changes in intracellular glucose concentrations rapidly reflects changes in extracellular concentrations [Squires *et al*. 2005]. In

response to increased glucose levels, beta cells depolarise and generate action potentials to secrete insulin, therefore acting as glucose sensors [Rorsman and Braun, 2012]. The triggering pathway is well characterised, and the cascade leading to exocytosis of insulin during this first phase of insulin release is primarily driven by circulating glucose levels.

Glucose enters beta cells by facilitated diffusion, through glucose transporters. GLUT2 is critical for glucose-stimulated insulin secretion [Thorens, 2015], which is coupled by glycolysis [Guo *et al*. 2012]. On entering the beta cell, glucose is phosphorylated by a highly specific and low affinity glucokinase [Squires *et al*. 2005], a hexokinase enzyme, to glucose-6-phosphate and undergoes glycolysis resulting in production of pyruvate and yield of adenosine triphosphate (ATP). The rise in ATP: ADP ratio causes closure of ATP-sensitive potassium (K_{ATP}) channels, resulting in membrane depolarisation. In response, L-type voltage-dependent Ca^{2+} channels open, causing a rapid influx of extracellular Ca^{2+} ions. These changes in extracellular Ca^{2+} concentrations are monitored by a calcium-sensing receptor found within pancreatic islets [Squires *et al.* 2000], which plays key roles in cell-to-cell communication enabling a co-ordinated insulin secretory response [Jones *et al*. 2007]. This rapid influx increases intracellular Ca^{2+} concentrations and triggers the exocytotic machinery, resulting in an increased rate of insulin secretion from the beta cell [Komatsu *et al*. 2013]. Therefore, pancreatic beta cells can be described as secretory cells, which have evolved mechanisms to link increased metabolic activity with increased electrical activity [Squires *et al*. 2005].

In second phase insulin release, metabolism of glucose produces signals that augment Ca^{2+} induced insulin exocytosis [Grespan *et al.* 2018]. This phase is a sustained, slow release of the newly formed vesicles [Grespan *et al.* 2018]. Under these conditions, glucose still increases insulin secretion in a concentration-dependent manner, as well as through a non-glucose mediated response [Henquin, 2000]. This pathway remains functionally silent as long as glucose has not reached its threshold and initiated an influx of Ca^{2+} ions during the first triggering pathway [Henquin, 2000].

Once released, insulin initiates its actions through binding to the alpha subunit on a glycoprotein cell surface receptor, stimulating the tyrosine-specific protein kinase of the beta subunit [Gammeltoft and Van Obberghen, 1986]. Activation of intrinsic tyrosine kinase activity initiates both the PI-3-kinase-dependent pathway, which is essential for GLUT4 translocation, as well as the CAP/Cbl complex pathway, and plays a vital role in insulin-stimulated glucose utilisation through a PI-3-kinase–independent pathway [Baumann *et al*. 2000]. In muscle, glucose uptake is fundamentally insulin-dependent through GLUT4 and promotes glycogen synthesis by activating glycogen synthase [Bouskila *et al*. 2008]. This enables the release, anaerobically, of energy by glycolysis. Insulin acts on the adipose tissue to promote glucose uptake, stimulates lipogenesis, but supresses lipolysis [Cignarelli *et al*. 2019]. In liver, insulin modulates lipoprotein metabolism and protein synthesis, as well as glucose storage and inhibits gluconeogenesis [Claus and Pilkis, 1976]. It has also been suggested that in the brain, insulin acts as a neuropeptide, playing roles in regulation of appetite, satiety, memory and cognition [Gerozissis, 2004].

However, characteristics of T2DM include the loss of first-phase and reduced second-phase secretion. A reduction in glucose-stimulated-insulin-secretion as well as impaired glucose tolerance are indicators of early T2DM. Therefore, this necessitates the development of novel therapeutics that help restore adequate secretion of insulin in T2DM [Henquin, 2000].

1.1.4 OBESITY AND TYPE 2 DIABETES MELLITUS

The WHO defines obesity as an "abnormal or excessive fat accumulation that presents a risk to health". Body mass index (BMI), calculated by dividing a person's weight (kg) by the square of body height (m^2), is a crude method in diagnosing obesity, with percentage body fat a more accurate method [Romero-Corral et al. 2008]. In comparison to a normal BMI range of 18.5 – 24.9 kg/m^2, a BMI of $\geq$ 30 kg/m^2 is generally considered as obese, with a BMI equal to $\geq$ 25 kg/m^2 classed as overweight [WHO, 2019]. Overweight and obesity was once considered a problem in only wealthier countries, however there have been dramatic increases in lower and middle-income countries. In 1995, the global obese population was estimated to be approximately 200 million adults [Golay and Ybarra, 2005]. However, in 2016, over 650 million adults were reported as obese, with over 340 million children, aged 5-19, classed as obese [WHO, 2018]. There are also strikingly similar observations with the prevalence of T2DM.

Various factors contribute to this global epidemic, for example environmental, food availability, geographic influences, genetic obesity-related traits [Frayling *et al*. 2007; Loos and Bouchard, 2008; Meyre *et al*. 2009; Speliotes *et al*. 2010], as well as individual characteristics, such as age, sex, race and ethnicity [Lee *et al*. 2019]. Due

to the escalating rates of obesity, other chronic disease risks are further exacerbated, as obesity is a major risk factor for osteoarthritis, hypertension, cancer, respiratory diseases, cardiovascular diseases and diabetes. It is estimated by the McKinsey Institute that overweight and obesity related problems cost US$2 trillion annually [Dobbs *et al*. 2014]. In the UK, it was estimated to cost the NHS £6.1 billion in 2014 -2015, with this projected to reach £9.7 billion by 2050 [Foresight, 2007], if we fail to address this epidemic. This increasing escalation in the global epidemic of obesity has triggered a parallel vertiginous rise in incidence and prevalence in T2DM diagnosis. Obesity and T2DM are closely linked, with obese individuals having a seven times greater risk of developing this disorder [Abdullah *et al*. 2010]. However, precise mechanisms on how the two conditions link still remain unclear. Recent studies have identified various links between obesity and T2DM, including pro-inflammatory cytokines (tumour necrosis factor and interleukin-6), insulin resistance, deranged fatty acid metabolism, and cellular processes such as mitochondrial dysfunction and endoplasmic reticulum stress [Kern *et al*. 2001; Blaak, 2003; Højlund *et al*. 2008; Cnop *et al*. 2012].

Studies have proposed various links between insulin resistance and obesity, which predispose to T2DM. This includes excessive fat deposition, particularly in the visceral area [Kahn, 2003], which reduce the ability of peripheral tissues to respond adequately to insulin. Also an increased production of adipokines and pro-inflammatory cytokines, including tumour necrosis factor-α, resistin, and retinol-binding protein 4, which worsen insulin resistance and reduce adiponectin levels [Deng and Scherer, 2010]. Furthermore, mitochondrial dysfunction, which decreases sensitivity to insulin and compromises the function of beta cells [Bournat and Brown,

2010]. Moreover, changes in the glucose-fatty acid balance contribute to insulin resistance and hyperglycaemia. In obesity, the increase in lipid oxidation due to elevated plasma free fatty acids results in insulin resistance, with escalation of this associated with development of diabetes. Due to insulin resistance, there is a compensatory mechanism in obese individuals, which aims to elevate secretion of insulin [Cavaghan *et al*. 2000] through increased beta cell mass and function [Chen *et al*. 2017]. Nevertheless, despite compensatory mechanisms of increased glycaemia and insulinaemia, the persistent insensitivity to insulin and resistance to glucose uptake in obese individuals gradually leads to T2DM and its associated complications.

Current therapeutics for T2DM, other than metformin, tend to be weight-neutral or associated with weight gain, which is highly unfavourable due to the major links aforementioned between obesity and T2DM. Therefore, there is pressing issue for novel therapeutics, as studies have demonstrated that even modest weight reductions can improve glycaemic control and reduce the risk of T2DM [Resnick *et al*. 2000; Wilding, 2014; Avery *et al.* 2019].

1.1.5 BETA-CELL DYSFUNCTION AND INSULIN RESISTANCE IN TYPE 2 DIABETES MELLITUS

The pathogenesis of T2DM is characterised by hyperglycaemia that is worsened by both beta cell dysfunction and insulin resistance, both inherently complex. Chronic exposure to hyperglycaemia leads to oxidative stress and inflammation, which induces changes in gene expression regulation and an increase in apoptosis [Gilbert and Liu, 2012]. Beta cell dysfunction, a critical determinant of T2DM [Ashcroft and Rorsman, 2012], results in impairment of insulin secretion, whereas with insulin

resistance, insulin can still be secreted but insulin insensitivity is established in target tissues [Cerf, 2013]. As these worsen, hyperglycaemia is augmented resulting in the progression of T2DM, associated with cytokine-induced inflammation, obesity, insulin resistance, overconsumption of free fatty acids and saturated fats [Cerf, 2013], with progressive beta cell decline leading to cell exhaustion and beta cell failure [Ferrannini, 2010; Talchai *et al*. 2012; Cerf, 2013]. This loss of beta cell mass and function is fundamental to the development of T2DM [Ferrannini, 2010].

An early physiologic indicator of beta cell dysfunction is a delay in the acute insulin response to glucose [LeRoith, 2002]. In T2DM, the first-phase response to elevated glucose concentrations is lost, resulting in excessive elevation of postprandial glucose. This ultimately leads to a hyperinsulinaemic second-phase insulin response to help dispose of this excess glucose [Porte and Kahn, 1995]. This compensatory mechanism occurs when integrity of the beta cells is weakened. If successful, normal physiology of the beta cells is maintained. However, if exhausted, beta cell dysfunction follows, with insulin resistance further impairing normal physiology and compensation thus exacerbating beta cell function [Cerf, 2013]. Others have suggested that the pathogenesis of this dysfunction may mimic hepatic steatosis: intra-tissue fat depots that induce inflammation thus triggering cell demise and dysfunction [Cerf, 2013]. Glucose toxicity and lipotoxicity also contribute to beta cell dysfunction, therefore if metabolic control is restored, this dysfunction could potentially be reversed [LeRoith, 2002].

Beta cell dysfunction is mediated by cytokine pro-inflammatory response, mitochondrial stress, ER and oxidative stress [Cerf, 2013]. In obese individuals, the

primary source of cytokines is through the infiltration of macrophages [Stienstra *et al*. 2007], with obesity also being a determinant of insulin resistance. However, the clear pathogenic mechanisms between obesity and insulin resistance still remains unclear, nevertheless numerous studies have suggested potential links between obesity, insulin resistance and T2DM. Obesity is a state of low-grade inflammation, and obesity-linked inflammation is found in adipose tissue and liver, coupled with elevated macrophage infiltration and pro-inflammatory cytokines [Ye, 2013]. This inflammation is associated with inhibition of insulin signalling pathways through various mechanisms, such as inhibition of IRS-1 and insulin receptors, inhibition of PPARγ function and accumulation of free fatty acids through triggering lipolysis [Ye, 2007; 2013]. In muscle, insulin resistance leads to impaired insulin stimulated glucose utilisation and decreased glycogen synthesis [Wilcox, 2005; Abdul-Ghani and DeFronzo 2010], whereas, as a result of accumulation of lipids in hepatocytes, liver inflammation is associated with hepatic steatosis, linked with insulin resistance. In obesity, insulin resistance is further exacerbated through intracellular fat deposition in muscle, liver and pancreas, resulting in fatty acids and glucose competing for uptake and metabolism in tissues [Cerf, 2013]. This elevation in FFA levels, which induce a glucolipotoxic state, coupled with persistent hyperglycaemia, is detrimental to pancreatic beta cell function and structure.

Elevated plasma insulin levels are a surrogate marker for insulin resistance [Greenberg and McDaniel, 2002]. In adipose tissue, increased expression of TNF-α has been shown to closely correlate with elevated fasting insulin levels. Hotamisligil and colleagues also demonstrated a direct link between expression of TNF-α and obesity-associated insulin resistance, with insulin resistance being ameliorated in obese rats

when TNF-α was neutralised [Hotamisligil *et al*. 1993]. Cytokines, such as TNF-α and IL-6, have also been shown to increase adipocyte lipolysis which in turn leads to elevated release of free fatty acids [Päth *et al*. 2001] which ultimately leads to the loss of insulin-secreting abilities [Boden, 1997].

1.2 CURRENT TREATMENT OPTIONS FOR T2DM

Non-pharmacological treatment for T2DM includes lifestyle and diet changes, to achieve normoglycaemia. However, lifestyle interventions are often not enough to achieve effective glycaemic control, and therefore pharmacological intervention is required. Table 1.1 summarises traditional treatment classes for T2DM, with metformin being the first-line therapy. Metformin, belonging to the class of antidiabetic agents known as biguanides, acts to improve insulin sensitivity and lower glucose production to enable more effective insulin utilisation, which can be used in combination with other drug classes that focus on stimulating insulin secretion. However, many of these conventional agents exhibit reduced efficacy over time, resulting in poor glycaemic control. There has also been a disappointing effect of these drug classes on weight loss, with older anti-diabetic therapies such as sulfonylureas and thiazolidinediones exacerbating weight gain. However, newer glucose-lowering agents including incretin mimetics and SGLT2 inhibitors have demonstrated weight-lowering effects [Pereira and Eriksson, 2019]. Therefore, various newer classes of drugs, discovered below, have been developed to help manage this epidemic.

Table 1.1: Classes of T2DM therapeutics

INSULIN SENSITIZERS			
Class	**Mechanism of Action**	**Advantages**	**Limitations**
• Biguanides – Example: Metformin	Stimulates AMP activated protein kinase [Klip and Leiter 1990] and inhibits adenylate cyclase [Bridges, 2014], to increase insulin sensitivity, suppress hepatic glucose output and to increase glucose uptake in the muscle [Zhou *et al*. 2001].	• Prevents weight gain • Reduces risk of hypoglycaemia • Decreased risk of associated complications [Viollet *et al*. 2012]	• Gastric problems • In patients with renal impairment, it can lead to lactic acidosis [Bailey, 1992].
• Thiazolidinediones - Example: pioglitazone	TZD's directly bind on the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), to regulate the transcription of various factors including glucokinase and GLUT-4 transporters	• Promote maturation of fat cells and deposition of fat into peripheral tissues. • Enhances beta cell function and sensitivity to insulin [Parulkar *et al*. 2001].	• Potentially severe side effects such as fluid retention, edema, weight gain, and heart failure [Kheirandish *et al*. 2017]. • Increase in peripheral fat mass
INSULIN SECRETAGOGUES			
Class	**Mechanism of Action**	**Advantages**	**Limitations**
• Sulfonylureas - Example: Tolbutamide	Binds to the sulfonylurea receptor (SUR1) on the beta cells, leading to the closure of ATP-dependant potassium (K^{ATP}) channels initiating cell depolarisation [de Wet and Proks, 2015], resulting in an influx of intracellular Ca^{2+} which promotes exocytosis of insulin.	• Stimulates insulin secretion	• Insulin secretion independent of plasma glucose levels, thus increased risk of hypoglycaemia • Weight gain • Increased risk of cardiovascular disorders [Li *et al*. 2014].
• Meglitinides - Example: Repaglinide	Binds to the benzamido site within the SUR1 receptors and influence the peroxisome proliferator activated receptor (PPARγ).	• Rapid • Stimulate insulin secretion • Improve insulin resistance	• Expensive • Short-acting – require frequent administration

1.2.1 SODIUM GLUCOSE CO-TRANSPORTER 2 (SGLT2) INHIBITORS

SGLT-2, present in proximal tubule of the nephron, assists in re-absorption of glucose in the kidneys. SGLT-2 inhibitors, such as canagliflozin and dapagliflozin, represent the most recently approved oral class of T2DM drugs. SGLT-2 inhibitors increase urinary excretion of glucose and reduce serum glucose levels [Vallianou *et al*. 2016]. Key advantages of SGLT-2 inhibitors include enhanced insulin secretion due to indirect effects on improved beta cell function, improved glycaemic control, weight loss and reduced blood pressure. SGLT-2 inhibitors promote urinary glucose excretion through inhibition of glucose reabsorption, resulting in related caloric reduction and projected weight loss. However, the expected weight loss is often not translated to the clinic, which suggests a compensatory increase in caloric intake during treatment with SGLT-2 inhibitors [Tentolouris *et al*. 2019]. Inhibition of SGLT-2 receptors have been shown to enhance glucagon secretion, through lowering intracellular glucose levels. This results in an increase in glucagon to insulin ratio, thus promoting lipolysis and ketogenesis [Tentolouris *et al*. 2019]. SGLT-2 inhibitors have also been shown to have a positive effect on the brain and cognitive activity [Lin *et al*. 2014]. SGLT-2 inhibitors are quickly becoming a favourable treatment option, but increase risks of contracting urinary tract infections [Bouchie, 2013].

1.2.2 INCRETIN MIMETICS

The incretin effect in patients with T2DM is severely reduced with this pathophysiological trait likely playing a vital role in the insufficient secretion of insulin to prevent hyperglycaemia after an oral glucose load in these individuals (Figure 1.1). The diminished incretin effect includes reduced GLP-1 secretion and reduced GIP action. Incretin mimetics are anti-diabetic agents that mimic the incretin

hormones, GLP-1 and GIP. Unlike GIP, the insulinotropic effect of GLP-1 is preserved in individuals with T2DM, therefore therapeutics have been modulated based around exploiting the beneficial actions of GLP-1. These agents mimic the anti-hyperglycaemic properties of GLP-1 by stimulating insulin secretion, reducing glucose uptake and inhibiting glucagon secretion [Nielsen, 2005; Gupta *et al*. 2016]. GLP-1 incretins approved for clinical use can be classed as either short acting (e.g. exendin-4), or long acting (e.g. liraglutide) [Uccellatore *et al*. 2015]. Exendin-4, the first incretin mimetic to be approved in 2005, is isolated from the saliva of the venomous lizard *Heloderma suspectum* [Eng *et al*. 1992]. This mimetic requires twice daily administration, and stimulates both first and second phase insulin secretion and has favourable effects on body weight [Szayna *et al*. 2000; Kolterman *et al*. 2003; Uccellatore *et al*. 2015]. Liraglutide, exhibits a similar beneficial effect to exendin-4, but only requires once daily injection. Weekly formulations of these GLP-1 mimetics have also been approved. These combine the GLP-1 agonists with either human albumin (Albiglutide) or with an IgG fragment (Dulaglutide) [Jimenez-Solem *et al*. 2010; Trujillo and Nuffer, 2014]. More recently, an oral GLP-1 mimetic has been FDA approved; Semaglutide [Dhillon, 2018].

Incretin mimetics are DPP-4 resistant. The use of these therapeutics have favourable advantages over aforementioned treatment options including better glycaemic control with reduced risk of hypoglycaemia, robust effects on weight loss, enhancements in beta cell function and improvements in patients' cardiovascular risk profile [Nauck, 2013]. Although incretin mimetics have been considered extensively for their beneficial effects for diabetes management, studies have also revealed beneficial attributes of these mimetics in various other tissues [Irwin and Flatt, 2015]. In bone,

data has suggested favourable anabolic effects [Mabilleau *et al.* 2014; 2015; Sun *et al.* 2015], however others contradict this and suggest that use of these mimetics could potentially increase fracture risk [Su *et al.* 2015]. Other studies have demonstrated improvements in cognitive function, and both cerebral and systemic microvascular architecture [Kelly *et al.* 2015] leading to improvements in learning and memory [Gault and Hölscher, 2008; Porter *et al.* 2011; Gupta, 2012], as well as promoting neuroneogenesis [Hamilton *et al.* 2011]. Like all therapeutics, some side effects have been reported including moderate nausea, vomiting and diarrhoea [Lund *et al.* 2014].

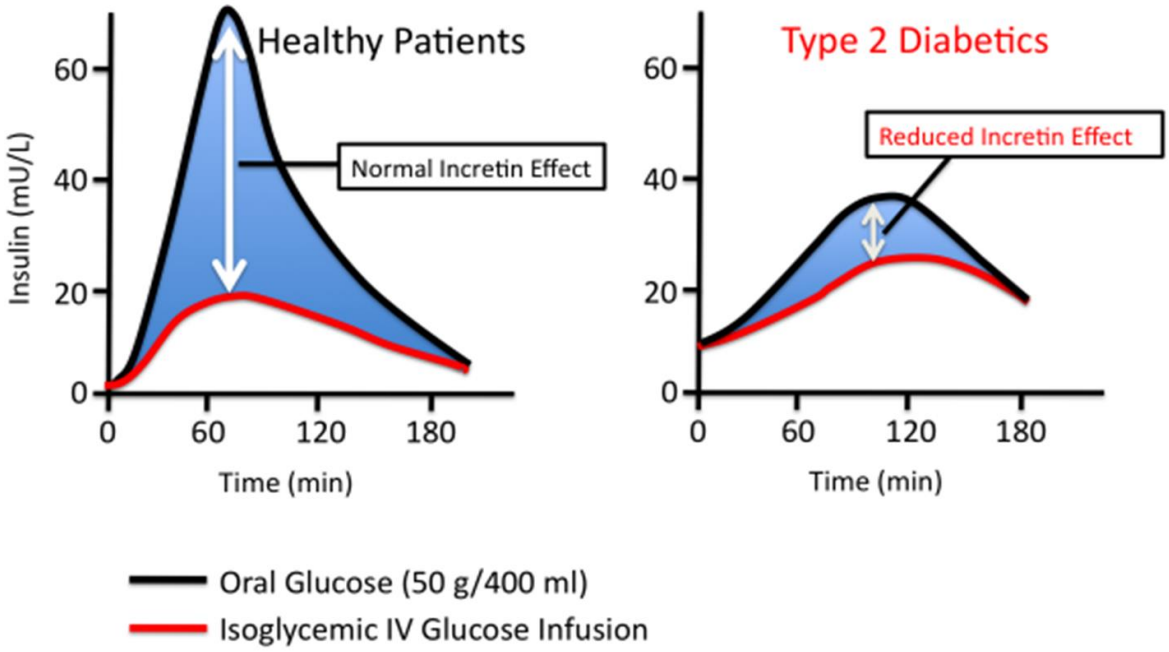


Figure 1.1 The incretin effect. Following an oral glucose load there is a stimulation of insulin secretion in healthy patients. However, in T2DM following an oral glucose load, this stimulation of insulin secretion is greatly impaired. [Taken from Nauck *et al.* 1986].

1.2.3 DIPEPTIDYL PEPTIDASE-4 (DPP-4) INHIBITORS

Dipeptidyl peptidase-4 (DPP-4) is an enzyme responsible for the degradation and subsequent short half-life of incretin hormones GIP and GLP-1 (Figure 1.2). DPP-4 rapidly cleaves and renders these incretin hormones inactive. Thus, to preserve the

impaired incretin effect, blockade of DPP-4 has been utilised as a mechanism to restore normal glycaemia in T2DM.

Clinically approved DPP-4 inhibitors, are a rationally designed drug class based on known endocrinology of GLP-1 and understanding of DPP-4 in metabolism, and include sitagliptin, saxagliptin, vildagliptin, alogliptin and linagliptin. By slowing the degradation of incretin hormones, these therapeutics result in higher circulating levels of active GLP-1 and GIP, which enhance their insulinotropic effects in T2DM patients [Green *et al*. 2004], as well as slowing gastric emptying, reducing food intake and reducing postprandial glucagon [Makrilakis, 2019].

Sitagliptin was the first clinically approved oral DPP-4 inhibitor and necessitates once-daily treatment. It is used in combination with other anti-hyperglycaemic therapeutics for the management of T2DM. The efficacy of sitagliptin has been firmly established in both animal models and in a broad spectrum of individuals with T2DM [Mu *et al*. 2006; Aschner *et al*. 2006; Williams-Herman *et al*. 2010; Katzeff *et al*. 2015]. DPP-4 inhibitors are not associated with an increased risk of hypoglycaemia or weight gain [Scheen, 2012] and are usually well tolerated, but are more expensive than some other available anti-diabetic therapeutics. Long term treatment with DPP-4 inhibitors in mouse models of obesity/diabetes, facilitates improved glycaemic control [Ahrén *et al*. 2004], beta-cell survival [Pospisilik *et al*. 2003], cognition [Gault *et al*. 2015b], as well as improving both cardiac and vascular endothelial stiffness [Aroor *et al*. 2014]. The benefits of DPP-4 inhibitors are mediated through the preservation of both incretin and non-incretin substrates, [Andersen *et al.* 2018], and not because of direct inhibitor action. Albeit, the many advantages of sitagliptin, safety concerns for clinical use have

been raised regarding potential increased risks of pancreatitis [Sue *et al*. 2013] and pancreatic cancer [Tseng, 2016]. However, with its convenient oral, once-daily routine, high efficacy and tolerability, as well as low risk of pharmacokinetic drug-drug interactions, sitagliptin still remains a key treatment option for T2DM patients.

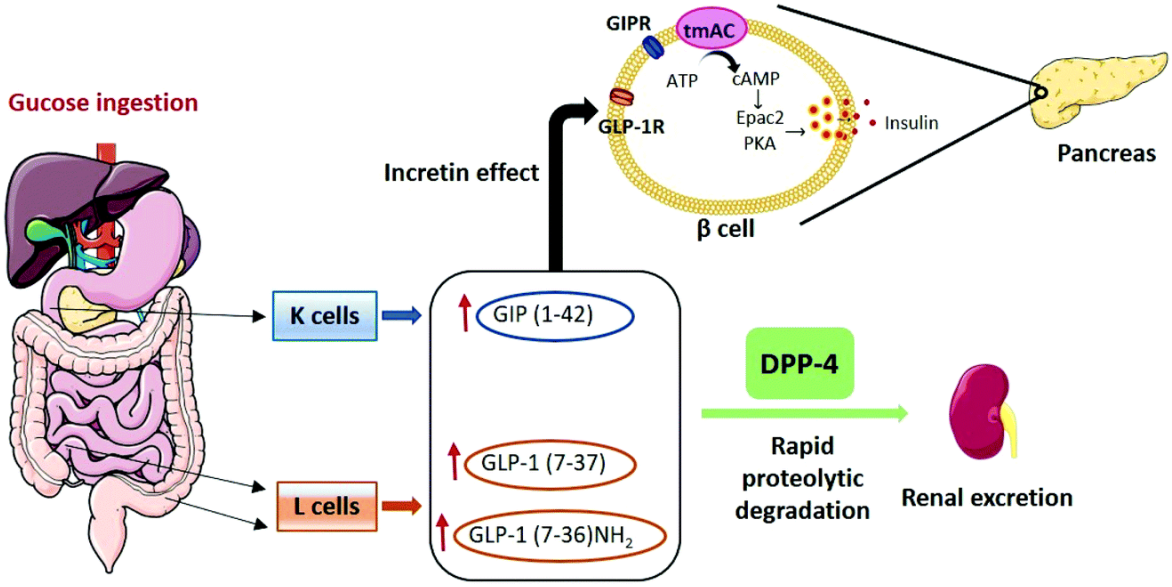


Figure 1.2 Metabolic Pathway of DPP-4. Following oral glucose, the incretin hormones GLP-1, secreted from intestinal L-cells, and GIP, secreted from intestinal K-cells are released. Upon binding to their respective receptors (GLP-1R and GIPR) in pancreatic beta cells, a cascade is triggered, resulting in increased cyclic adenosine monophosphate (cAMP) concentrations and subsequent activation of protein kinase A, potentiating glucose-stimulated insulin secretion. GIP and GLP-1 have relatively short half-lives due to their rapid degradation by the proteolytic enzyme DPP-4. However, treatment with DPP-4 inhibitors can hinder this process, thus augmenting the incretin effect and subsequent glucose-dependent insulin secretion, increasing glucose uptake in the muscle, decreasing glucose production in the liver, leading to reductions in blood glucose levels. [Taken from Proença *et al*. 2019].

1.2.4 BARIATRIC SURGERY

The gastrointestinal tract plays a key role in metabolic regulation and control, thus many treatment paradigms act to target the gastrointestinal tract to manage T2DM. Moreover, due to obesity presenting as a risk factor for diabetes, weight loss is also a

key target in the management of T2DM. Bariatric surgery not only results in significant weight loss and beneficial effects on glucose homeostasis, insulin secretion and sensitivity, but has been shown to result in T2DM remission [Cefalu *et al.* 2016]. Primarily, bariatric surgery improves beta-cell function through enhanced insulin sensitivity, and increases circulating levels of GLP-1 and GIP, which sequentially promote insulin secretion. The surgery also promotes the secretion of other gut hormones, such as PYY and the enteroglucagon family of peptides [Vincent and Le Roux, 2008], thus affecting metabolism, food consumption and satiety effects [Holst *et al*. 2018]. However, bariatric surgery is not an available option to all patients, and therefore novel therapies, which can replicate the success of this surgery is required.

1.2.5 INSULIN THERAPIES

When diet, lifestyle changes and other anti-diabetic agents have been optimally used, insulin therapy is often required. Previously, insulin therapy was used as a last resort therapy in T2DM, however it is now often prescribed earlier, and can be used as an initial 'rescue therapy' to help stabilise glycaemia. The preferred methods of insulin initiation in patients with T2DM include long-acting basal insulin, once-daily formulations or in combination with GLP-1 receptor agonists or other orally available antidiabetic drugs [Silver *et al.* 2018].

1.2.6 NEED FOR NOVEL ANTIDIABETIC DRUG DEVELOPMENT

Current therapies used to manage T2DM are primarily synthetic pharmacological drugs, which aim to improve insulin secretion, sensitivity to insulin and for glucose control. They are generally well tolerated in patients and can be effective in managing T2DM. However, despite the plethora of available drugs, many are ineffective in a

number of patients, with a high failure rate of current therapies and resultant side effects [Nyenwe *et al.* 2011]. Moreover, due to the increasing prevalence of this disorder [IDF, 2019], cost and drug availability are becoming an increasing concern. Therefore, there is a continuous and rapid need to develop novel therapeutics that are effective, affordable and globally available to manage T2DM.

1.3 PEPTIDES THERAPEUTICS

Peptides, with a relatively low molecular weight, can be used as unique pharmaceutical compounds, which have evolved as highly effective signal transduction molecules capable of exerting significant physiological effects [Fosgerau and Hoffmann, 2015]. Peptides as therapeutics are generally well tolerated and are regarded as safe, in addition to being highly selective and efficacious [Fosgerau and Hoffmann, 2015]. Thus, there is an increased interest into the research and development of peptides as potential therapeutics. Currently over 60 peptide drugs have been approved for use, 260 peptide drugs have been tested in human clinical trials, with a further 150 currently in active clinical development [Lau and Dunn, 2018]. The global market for peptide therapeutics is continually expanding, with $30 billion spent in 2018, however this is estimated to surpass $50 billion by 2024. This significant increase into novel peptide therapeutics has been triggered by the high prevalence of lifestyle disorders like obesity and T2DM and their associated side effects.

1.3.1 PEPTIDES AS DRUGS

The use of peptides as drugs has long been recognised as a significant opportunity, since the use of small molecules can be challenging when addressing certain disease

targets. Peptides used as therapeutic agents are normally derived from natural sources and termed bioactive peptides; however, peptides can also be isolated from chemical or genetic libraries. As peptides are naturally occurring, they have many advantages over small molecules including cheaper to synthesise, diverse, safer, with minimal risk of drug-drug interactions [Marqus *et al*. 2017], high binding affinity towards a broad range of therapeutic targets, and very potent resulting in enhanced efficacy, selectivity and specificity, even at lower doses [Fosgerau and Hoffmann, 2015]. Therefore, peptides are of high interest for many drug developers. However, peptides as drugs only represent ~2% of the drug market, whereas small molecules compile ~80%, with enhanced stability, oral bioavailability and permeability over unmodified peptides, but they may increase the risk of toxicity.

Unfortunately, despite their numerous advantages, clinical use of these peptides is hampered due to certain drawbacks limiting their therapeutic practice. These limitations comprise increased proteolytic instability, reduced oral bioavailability, limited permeation across biological barriers due to high hydrogen bonding capacity and lipophilicity [Rezai *et al*. 2006], susceptibility to enzymatic degradation as well as rapid renal clearance [Di, 2015]. Unmodified peptides are also usually limited to extracellular targets due to poor cell membrane penetration [Di, 2015]. Peptides have poor oral bioavailability and are normally administered either by subcutaneous, intramuscular or intravenous injections.

Importantly, these limitations can be overcome as various peptides have shown effectiveness for numerous disease states such as peptide-derived vaccine for prostate cancer [Perez *et al*. 2010], as well as other anticancer agents [Lehmann *et al*. 2006;

Marqus *et al*. 2017], management of irritable bowel disease with the peptide compound linaclotide [Bassotti *et al*. 2018], and the clinically approved GLP-1 receptor agonist exendin-4, isolated from the salvia of the Gila monster *Heloderma suspectum* [Eng *et al*. 2002] for management of T2DM.

1.3.2 APPROACHES TO IMPROVE PEPTIDE PHARMACOKINETICS AND PHARMACODYNAMICS

The pharmaceutical industry has taken considerable interest in development of peptides as novel drug candidates, due to their impressive physiological and biochemical effects, as well as subjugating the afore-mentioned limitations. This has included various approaches to improve both the pharmacokinetic and pharmacodynamic profile of peptide-based therapeutics with modified peptides or peptidomimetics. These approaches consist of maintaining or enhancing biological activity, whilst altering the peptides physiochemical characteristics [Gentilucci *et al*. 2006].

Peptides have a shorter biological half-life due to their susceptibility to enzymatic degradation and rapid renal clearance, consequently resulting in reduced peptide efficacy due to reduced exposure to target tissues. Therefore, to overcome this problem, various approaches are being established to increase peptide half-life. This includes structural modification of the N-terminal/C-terminal of peptides such as amidation, acetylation or glycosylation to enhance stability, as well as cyclization to enhance peptide stability and rigidity [Werle and Bernkop-Schnürch, 2006; Goodwin *et al*. 2012; Di, 2015]. Amino acid substitutions, including the replacement of naturally occurring L-amino acids with unnatural D-amino acids forms, have been shown to

increase peptide stability, as very limited enzymes have been discovered to degrade D-amino acids [Feng and Xu, 2016]. Peptide half-life can also be increased through the covalent attachment of polyethylene glycol (PEG) chains to peptides. This also prevents the recognition and degradation of peptides by proteolytic enzymes. Furthermore, due to the now increased peptide size by PEG, renal filtration is reduced and peptide bio-distribution altered, as well as decreased immunogenicity and antigenicity of the peptides [Veronese, and Pasut, 2005; Veronese and Mero, 2008]. Increasing peptide molecular size, through conjugation with gamma immunoglobulin (IgG), can also result in increased uptake of the peptide at the biological active site [Bruno *et al*. 2013]. Attachment of fatty acids to peptides has also been shown to increase their half-life by reducing glomerular filtration via albumin binding [Madsen *et al*. 2007]. Albumin is the most abundant blood serum protein, with a biological half-life of 19 days [Peters, 1996], therefore the use of ligands as carrier systems, which carry the peptide onto longer-lived serum proteins like albumin, helps to enhance peptide half-life [Larsen *et al*. 2016]. Peptide drugs, which contain albumin-binding fatty acids in their structure, include liraglutide, which due to the retardation of renal elimination due to albumin binding, gives liraglutide a half-life of 12 hours [Krentz and Sinclair, 2012]. Covalent linkage of peptides with the serum protein albumin or IgG fragments can reduce renal clearance; extend peptide half-life whilst retaining peptide potency. For example, albiglutide, a GLP-1 mimetic, which has an increased half-life of 5-days, enabling a once weekly injectable drug [Rosenstock *et al*. 2009]. Peptide half-life can be enhanced through the introduction of reduced peptide bonds, resulting in a peptide highly resistant to enzymatic and chemical degradation [Feurle *et al*. 2003]. Bio-stability of peptides can be further improved through the inclusion of disulphide bridges, which have been shown to be critical in both the folding and

structural stabilisation of peptides [Chen *et al*. 2001]. Half-life of peptides can also be increased using sustained release techniques, this includes the use of matrix-free formulation, such as in taspoglutide [Rosenstock *et al*. 2013], or through the incorporation of polylactide-glycolide microsphere suspension with peptides, for example exenatide-LAR [Drucker *et al*. 2008].

To help further the efficacy of peptide therapeutics, approaches to increase peptide bioavailability have been used. Through the addition of modules enabling passive or active transport, peptide drug penetration through biological barriers can be improved [Fasano, 1998]. This includes the use of positively charged amino acids, especially at terminal positions, to improve cell and tissue penetration [Teesalu *et al*. 2009; Li and Cho, 2012], as well as the incorporation of sugars which can improve tissue penetration including transport across the blood-brain barrier [Otvos and Wade, 2014]. Conjugation of peptides with ligands of various cell surface receptors can also help improve penetration of peptides. Intracellular transport of peptide drugs can be further enhanced through the fusion with highly cationic low molecular weight penetrating peptides [Lindgren and Langel, 2011].

Increasing peptide solubility can also help improve peptide drug efficacy. Solubility can be increased in peptide formulations through the incorporation of salts, sugars or other compounds such as heparin [Uhlig *et al*. 2014]. A successful peptide dosage formulation requires sufficient solubility, dependent on the pH of the peptide solution, to allow for a dosing volume suitable for the selected route of administration [Bak *et al*. 2015]. Peptide solubility can be increased via pH adjustment away from the

peptide's isoelectric point. Tolerable range of intravenous administration is pH 3–10 [Bak *et al*. 2015].

The route of administration largely affects the pharmacokinetics of peptide-based drugs, with parenteral routes commonly used for peptide drug administration. However, more patient friendly routes of administration have been a recent research focus, with oral delivery being the most pursued. Many advances to accomplish oral delivery includes the use of carriers such as nanoparticles, microspheres and liposomes [Muheem *et al*. 2016; He *et al.* 2019], as well as the use of absorption enhancers such as bile salts, which enhance penetration and improve the bioavailability [Brayden and Mrsny, 2011; Sizer, 1997]. Previous studies demonstrated the potential of conjugating biotin with GLP-1 for oral drug delivery [Chae *et al*. 2007]. However, it was not until 2019, when the first oral GLP-1 mimetic was clinically approved by the FDA, using a stable and soluble carrier, SNAC (Sodium N-[8-(2-hydroxybenzoyl)Amino]Caprylate), which chaperones the GLP-1 mimetic, semaglutide, through the gastrointestinal membrane, thus overcoming issues with bioavailability without interrupting the therapeutic agent [Bucheit *et al*. 2020]. Metabolic profile of peptide drugs can also be improved. To better understand the pharmacokinetic profile of peptide drugs, absorption, distribution, metabolism and excretion have to be evaluated. Assessment of biological activity of peptide-based therapeutics can be assessed both *in vitro* and *in vivo*.

In addition, and specifically for the gut peptide xenin, given that other related insulin-releasing peptides, for example neurotensin, xenopsin and caerulein, possess an N-terminal pyroglutamic acid (pGlu) residue, which appears to prolong biological half-

life [Zahid *et al*. 2011], this approach may prove favourable for the augmentation of bioactivity of xenin and related fragment peptides. These methods outlined above result in the improvement of both the pharmacokinetic and pharmacodynamic profile of peptide drug candidates, consequently enhancing their therapeutic promise. The versatility, effectiveness, safety and tolerability of peptide-based candidates, due to the above approaches, is resulting in the increased translation to peptide-based therapies by several pharmaceutical industries, predominantly in the management or treatment of obesity and diabetes.

1.4 GUT PEPTIDES

In 1969, the concept of using gut-derived hormones as therapeutics for metabolic conditions was first considered [Unger and Eisentraut, 1969]. These early studies explained how food consumption stimulates release of various gut hormones that regulate the secretion of insulin [Drucker, 2006]. Various bioactive peptides and hormones are produced by the gut, such as GIP, GLP-1, xenin, ghrelin, oxyntomodulin, PYY, cholecystokinin (CCK) and gastrin [Feurle, 1998; Drucker, 2006; Wren and Bloom, 2007]. These active peptides and hormones are produced by endocrine cells within the gastrointestinal system, and can be released into circulation following food intake [Ahrén, 2003].

Previous studies highlighted the ability of interaction of gut peptides to stimulate insulin secretion, particularly following food ingestion. CCK, gastrin, GIP and GLP-1, could play vital roles in regulating insulin secretion and lowering blood glucose levels [Dupre *et al*. 1973; Ahrén *et al*. 2000; Drucker, 2007; Rehfeld, 2017], whilst also having effects on suppression of appetite and increasing energy expenditure [Tan

and Bloom, 2013]. Thus, due to defects in secretion of insulin in T2DM, the insulinotropic capabilities of these gut peptides may be promising targets for the treatment of this disorder. As such, direct modulation of one or multiple peptide pathways may prove useful for treatment of obesity/T2DM. Direct targeting of GLP-1 has already been established as a therapeutic agent for T2DM [Doyle and Egan, 2007], as well as research into gut hybrid peptides targeting multiple signalling pathways [Fosgerau *et al*. 2013; Irwin *et al*. 2013a, 2015; Bhat *et al*. 2013; Hasib *et al*. 2017, 2018; Craig *et al*. 2020]. However, further investigations are required to establish the role of the other related gut hormones, either alone but particularly in combination, as potential therapeutic agents for the treatment and aetiology of T2DM.

1.4.1 IMPAIRED INCRETIN EFFECT IN DIABETES

The incretin effect is defined as the increased stimulation of glucose-dependent insulin secretion elicited by oral as compared with intravenous administration of glucose under similar glucose conditions [Holst and Ørskov, 2004]. The incretin effect is mediated by gut-derived incretin hormones, GLP-1 and GIP, which are responsible for approximately 50-70% of postprandial insulin secretion [Nauck *et al*. 1986]. GLP-1 and GIP are confirmed incretin hormones in man [Irwin *et al*. 2019], and are secreted by enteroendocrine cells in the intestinal mucosa. Normally, the circulatory levels of these hormones increase following nutrient intake and stimulate glucose-induced insulin secretion [Nauck and Meier, 2018]. However, in T2DM this incretin effect is severely diminished and patients exhibit an almost complete loss of incretin hormone insulinotropic actions, which therefore contributes to the pathogenesis of this metabolic disorder.

This deficiency of the incretin effect could be due to the decreased secretion of GLP-1 and loss of second phase stimulation of the insulinotropic actions of GIP [Nauck *et al*. 1993; Holst *et al*. 2004; Holst *et al*. 2011], or impaired effects in pancreatic islet function. Compared to GLP-1, the reduced insulinotropic actions of GIP may be attributed to downregulation of GIP receptors (GIPR) [Holst *et al*. 1997]. Various studies have demonstrated the decreased expression of these receptors in both *in vitro* and *in vivo* hyperglycaemic states [Lynn *et al*. 2003, Xu *et al*. 2007; Pathak *et al*. 2015], showing the effects of elevated glucose levels and GIPR expression.

1.4.1.1 GLUCAGON-LIKE-PEPTIDE-1 (GLP-1)

The peptide hormone, GLP-1, acts as an incretin to stimulate release of insulin from beta cells in response to oral glucose. GLP-1, a 30-amino-acid hormone, is secreted from enteroendocrine L-cells in the small intestine [Herrmann *et al*. 1995]. GLP-1 is a posttranslational product of proglucagon, the precursor of glucagon [Orskov *et al*. 1989]. The 180-amino-acid preproglucagon is cleaved, forming the 160 amino acid proglucagon, which is cleaved to GLP-1 by prohormone convertase 1/3 (PC1/3) in intestinal L-cells, and by PC2 in pancreatic alpha cells [Whalley *et al*. 2011]. There are two major biologically active forms of GLP-1: GLP-1(7-36) amide and GLP-1(7-37). Native GLP-1 is rapidly degraded by DPP-4 with only 8% of newly secreted intact GLP-1 reaching peripheral circulation [Hjøllund *et al*. 2011]. This results in inactive metabolites and a short biological half-life, less than 2 minutes in rodents [Kieffer *et al.* 1995] and approximately 7 and 5 minutes in healthy subjects and individuals with T2DM, respectively [Deacon *et al.* 2000], therefore rendering the native form unsuitable for therapeutic use.

The actions of GLP-1 are primarily mediated through binding to G-protein coupled receptors (GLP-1R) [Thompson and Kanamarlapudi, 2013], which are widely expressed in the pancreatic islets, heart, stomach, intestines, lung, kidney, pituitary, hypothalamus and brain stem [Holst, 2007; Baggio and Drucker, 2007], as well as areas related to appetite regulation [Turton *et al*. 1996]. The main target for GLP-1 is the pancreatic islets, but GLP-1 also has roles in modulating the activity of other organs [Holst, 2007]. These pancreatic and extra pancreatic actions are summarised in Table 1.2. The main function of GLP-1 is to act as an incretin hormone, with its ability to augment postprandial insulin production being the most studied physiological effect of GLP-1.

Table 1.2: Biological actions of the incretin hormone GLP-1

Tissue:	**Effects**
Pancreas	• Increases insulin and somatostatin secretion • Decreases glucagon secretion • Increases insulin gene expression • Increase beta-cell sensitivity to glucose • Increase beta-cell proliferation and neogenesis • Decreases beta-cell apoptosis
Adipose	• Increases glucose uptake • Increases lipolysis
Stomach	• Delays gastric emptying • Decreases gastric motility • Reduces gastric acid secretion
Brain	• Increase satiety and energy expenditure • Reduces appetite • Promotes neuroprotection
Heart	• Increases glucose uptake • Promotes cardiac function and protection
Liver	• Decreases hepatic glucose production
Muscle	• Increases glucose uptake • Favourable effects on muscle blood flow
Bone	• Promotes bone formation • Reduces bone resorption

1.4.1.2 GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP)

GIP(1-42) is synthesised from its 153 amino acid precursor; ProGIP, by prohormone convertase PC1/3 [Kim and Egan, 2008], and to GIP(1-30) in the intestine and pancreatic alpha cells by PC2 [Fujita *et al*. 2010]. The enzyme DPP-4 rapidly degrades GIP, through cleavage at the N-terminal Ala^2 position, producing GIP(3-42), a GIP receptor antagonist [Gault *et al*. 2002a], thus GIP has a very short half-life of 2-5 minutes in circulation [Tatarkiewicz *et al*. 2014]. The incretin hormone GIP consists of three bioactive domains; GIP(1-14) - the N-terminal domain, GIP(19-30) - the mid domain, and GIP(31-42) - the C-terminal domain [Hinke *et al*. 2001]. The N-terminal and mid domains are involved with the insulin-releasing incretin effect, whereas the C-terminal domain is known for its enterogastrone activity [Hinke *et al*. 2001].

GIP, a 42-amino acid incretin hormone first isolated in 1969, is secreted from enteroendocrine K-cells in the intestinal mucosa in response to oral glucose [Nauck *et al*. 2004]. The established actions of GIP include, but are not limited to, stimulating glucose-induced insulin secretion, regulating postprandial glucose homeostasis, slowing gastric emptying and promoting pancreatic beta cell proliferation and survival [Irwin and Flatt, 2013]. Various studies in both rodents [Gault *et al*. 2003b] and humans [Gasbjerg *et al*. 2019], have established GIP as the major incretin hormone, which accounts for approximately 70% of the full incretin effect [Gault *et al*. 2003c; Holst, 2019]. In the pancreas, various studies have demonstrated the direct effects of GIP on beta cells to stimulate the secretion of insulin, protect against apoptosis [Taminato *et al*. 1977; Adrian *et al*. 1978], and enhance beta cell proliferation through the stimulation of PKA, PKB, MAPK and P13K pathways [Trümper *et al*. 2001]. GIP has other extra pancreatic actions including actions to increase lipogenesis and reduce

lipolysis in adipose tissue [Gupta and Raja, 2019], favourable cognitive effects including increase in proliferation of progenitor cells in the brain and positive effects on memory [Faivre *et al*. 2012], improving glucose utilisation in the liver [Chiang *et al.* 2012], and augmenting bone formation/decreasing resorption [Nissen *et al*. 2014].

Nevertheless, clinical exploitation of GIP has been hindered due to its well-characterised reduced action in T2DM, however, both GIP agonism and antagonism have both been associated with T2DM therapies. Numerous GIP-receptor agonists have shown insulinotropic potential and favourable anti-hyperglycaemic effects [Gault *et al*. 2003a; O'Harte *et al*. 2002; Irwin *et al*. 2005a, 2005b, 2006a, 2006b]. However, increasing concentrations of GIP have also been associated with promoting obesity [Thondam *et al*. 2017], due to the ability of GIP to stimulate fat synthesis [Fulurija *et al*. 2008]. Accordingly, more interest has been shown in GIPR antagonists, which have been described to reduce body weight and favourable changes in triglyceride levels [Kim *et al*. 2012; McClean *et al*. 2008]. However, analogues of GIP may yet offer a novel class of drugs for the management of diabetes, as in healthy individuals GIP seems to be the major incretin contributor [Bastin and Andreelli, 2019]. Due to the synergistic relationship between GLP-1 and GIP on beta cells, simultaneous activation of both pathways may provide additional benefits on beta cell functions. Bastin and Andreelli [2019], also suggest that through the enhancement of reduced blood glucose levels by GLP-1, this in effect promotes GIP activity to achieve improved metabolic control.

In patients with T2DM, the biological activity and glucose-induced insulin secretory action by GIP is greatly impaired, which is detrimental to the function of beta cells.

The primary pathophysiological source of this impairment is thought to be caused by the desensitisation and downregulation of the GIP-receptor in the pancreas [Knop *et al*. 2007b], caused by splicing of the mRNA resulting in decreased activity of the GIP-receptor [Gupta *et al*. 2017]. This diminished incretin effect is thought to be an early defect in T2DM, second to the development of insulin resistance [Muscelli *et al*. 2008]. Due to its weak efficacy and biological activity, this native 42-amino acid hormone exhibits unfavourable pharmacokinetic properties for the treatment of T2DM [Irwin *et al*. 2006a, 2006b]. However, due to the favourable insulinotropic actions of GIP which could be restored, including not only augmentation of the incretin effects, but also to protection against further decline in beta-cell mass; an underlying pathophysiological problems in diabetes, it would be highly beneficial if the actions of GIP were re-sensitised in patients with T2DM.

1.4.2 XENIN

Xenin, a 25 amino acid peptide, is the human counterpart of the amphibian peptide xenopsin [Araki *et al*. 1975], synthesised from its 35-amino acid precursor pro-xenin through the posttranslational enzymatic action of cathepsin-E [Kageyama, 1995; Hamscher *et al*.1996; Feurle, 1998]. Interestingly, this precursor proxenin has been described with sequence homology to yeast and mammalian alpha coat protein (COPA) [Hamscher *et al*. 1996], with all 35 amino acid residues of COPA identical to that of proxenin, with treatment of proxenin with pepsin releasing the biologically active xenin-25 [Chow and Quek, 1997; Craig *et al*. 2018]. It is a naturally occurring peptide hormone originally identified by Feurle and colleagues from isolates of human gastric duodenal and jejunal mucosa [Feurle *et al*. 1992], with subsequent studies further demonstrating that xenin-25 can similarly be extracted from the gastric mucosa

of various other animal species, including rat, rabbit, dog and pig [Hamscher *et al*. 1995; Craig *et al*. 2018]. In humans, xenin-25 is synthesised and secreted into the bloodstream from a subpopulation of chromogranin A-positive enteroendocrine K-cells [Anlauf *et al*. 2000], similar to GIP, in response to food intake [Taylor *et al*. 2010, Martin *et al*. 2016, Craig *et al* 2018]. A specific receptor for xenin is yet to be identified, but it is believed that due to the structural similarities between xenin-25 and neurotensin, some biological effects may be mediated through activation of neurotensin receptors [Clemens *et al*. 1997; Craig *et al*. 2018]. However, there is suggestions that some biological effects of xenin-25 may be independent of these receptors [Heuser *et al*. 2002].

The biological actions of xenin-25, summarised in figure 1.3, have been well established including effects on appetite suppression [Alexiou *et al*. 1998; Leckstrom *et al*. 2009; Chowdhury *et al*. 2014; Martin *et al*. 2016; Craig *et al*. 2018] gastrointestinal transit rate and gastric emptying [Feurle *et al*. 1992; Cline *et al*. 2007; Kim and Mizuno, 2010; Craig *et al*. 2018] as well as pancreatic endocrine and exocrine secretions [Feurle *et al*. 1992; Silvestre *et al*. 2003; Martin *et al*. 2016; Craig *et al*. 2018]. These actions of xenin are outlined in more detail below.

Appetite Suppression

Similar to other anorectic gastrointestinal peptides, circulating levels of xenin-25 increase following food intake, thus in harmony with this xenin-25 acts to control energy intake by acting as a satiety factor. [Feurle *et al*. 2003; Leckstrom *et al*. 2009; Cooke *et al*. 2009; Craig *et al*. 2018]. Indeed, Mrózek *et al*. 2012 demonstrated the regulation of energy balance by xenin-25 in children, revealing that the overall affect

may be altered by both intestinal inflammation and obesity. The clear anorexigenic effects of xenin-25 have been confirmed in both chicks [Cline *et al*. 2007] and rodents [Alexiou *et al*. 1998; Leckstrom *et al*. 2009; Cooke *et al*. 2009; Taylor *et al*. 2010; Craig *et al*. 2018]. The effects on feeding by xenin-25 may be directly associated with the actions on gastrointestinal transit as outlined below [Kim and Mizuno, 2010; Chowdhury *et al*. 2014], as decreased food consumption and a 93% reduction in the rate of gastric empting in xenin-25 treated mice was demonstrated by Kim and Mizuno, 2010. However, it has also been suggested that the anorexia inducing impact of xenin-25 in chicks is centrally mediated through the direct effects on ventromedialis hypothalami [Nandar *et al*. 2008]. Similarly, others have demonstrated these effects are also linked with the activation of cells in the nucleus of the solitary tract [Kim and Mizuno, 2010].

Xenin-25 may also affect feeding, independent of the actions of both melanocortin and leptin, by modulating neurotensin receptor related signalling pathways in the hypothalamus [Leckstrom *et al*. 2009; Kim *et al*. 2016]. It is believed by others that activation of these hypothalamic neurotensin receptors helps to control food intake through the stimulation of extracellular signal regulated kinase activity [Morikawa *et al*. 2004]. However, the suppressive effects of xenin-25 on appetite by extracellular signal regulated kinase pathways is also deliberated by others [Kim *et al*. 2016]. IL-1 signalling has also been identified as another potential central pathway through which energy balance is modulated by xenin-25 [Kim *et al*. 2014]. Indeed, it has also been suggested that myenteric neurons may also mediate the effects on feeding by xenin-25, along with xenin-25 effects on gastric emptying, gall bladder contractions, and gut motility [Zhang *et al*. 2012; Craig *et al*. 2018]. More recently, direct effects of xenin-

25 have been described on lipid metabolism and adipose tissue, including an increase in lipolysis and reduction in lipogenesis [Bhavya *et al*. 2017], which may also link to the notable effects on energy turnover by xenin-25 [Craig *et al*. 2018].

Gastrointestinal transit

The delay in gastric emptying is evident in both rodents and humans by xenin-25 [Kim and Mizuno, 2010; Chowdhury *et al*. 2014], with this effect thought to be linked to the presence of neurotensin receptors on nerve fibres in longitudinal stomach muscle [Chowdhury *et al*. 2014]. Extensive studies have outlined the actions of xenin-25 in both the small and the large intestines of guinea pigs [Feurle *et al*. 1996]. In the jejunum, a biphasic response to xenin-25 is elicited resulting in a small relaxation followed by a large contraction. This is assumed to be caused by a neurokinetic excitatory effect, along with muscarinic purinergic and tachykinin-related mechanisms [Feurle *et al*. 1996]. In the colon, xenin-25 induces a myokinetic relaxation effect, which involves the calcium-dependent potassium channels and the P2-purinoceptor [Feurle *et al*. 1996]. These distinct differences in the action of xenin-25 in the intestines of guinea pigs, may suggest there are variants in both neurokinetic and myokinetic receptors responsible for these effects by xenin-25 [Craig *et al*. 2018]. Additionally, in the rat ileum, Clemens *et al*. 2007, reported that this effect is mediated by an apamin-sensitive neurotensin-type receptor. In dogs, a dose-dependent neurotensin-like effect on gall bladder contractions, with a non-dose dependent effect on jejunum contractions was elicited by xenin-25 [Kamiyama *et al*. 2007].

However, others have also proposed that the effects of xenin-25 may not be entirely mediated through the interaction with neurotensin receptors on the intestine [Heuser

et al. 2002], but suggest the presence of other receptors which can regulate the activity of xenin-25 [Mazella *et al*. 2012]. Following Roux-en-Y gastric bypass, recent studies have demonstrated the absence of xenin-25 stimulated gastrointestinal transit [Kaji *et al*. 2017], indicating the importance of the duodenum in this regard [Craig *et al*. 2018]. In humans, interdigestive and postprandial duodenojejunal motility is also modulated by xenin-25, demonstrated at relatively low infusion doses of 40 ρmol/kg for 10 min [Feurle *et al*. 2001]. Feurle and co-workers also demonstrated the inhibition of pentagastrin-stimulated gastric acid secretions by xenin-25, which could also be a factor in the modulation of overall gastric transit [Feurle *et al*. 1997].

Pancreas

Initial studies by Feurle *et al*. 1992, showed that xenin-25 stimulated secretions from the exocrine pancreas, with the same research team presenting that, in dogs, xenin-25 also induces secretion of pancreatic-derived endocrine hormones, including insulin, glucagon and pancreatic polypeptide [Feurle *et al*. 1997], which was later confirmed in isolated mouse islets [Silvestre *et al*. 2003]. These studies would therefore suggest that the effects on glucagon and insulin release by xenin-25 is mediated through a direct effect on the pancreatic alpha and beta cells, respectively [Craig *et al*. 2018]. In contrast, it has also been suggested that xenin-25 does not act directly on the pancreatic beta cells, but instead acts on non-ganglionic cholinergic neurons to stimulate acetylcholine release resulting in the activation of beta cell muscarinic receptors [Wice *et al*. 2010]. However, it has been reported that these cholinergic pathways are not involved in the stimulatory effects on pancreatic secretions by xenin-25 [Nustede *et al*. 1999]. Furthermore, studies have also suggested that the insulinotropic actions of xenin-25 are not mediated through activation of neurotensin receptors in mice [Taylor

et al. 2010]. More recent studies have also highlighted the potential of xenin-25 as a direct independent insulinotropic agent, as well as its ability to potentiate both insulin-releasing and glucose-lowering actions of GIP, the incretin hormone [Taylor *et al*. 2010; Wice *et al*. 2010, 2012; Martin *et al*. 2012, 2014; Gault *et al*. 2015; Parthsarathy *et al*. 2016]. Additionally, xenin-25 has been shown to enhance proliferation in pancreatic beta cells in both rodent and human beta cells [Khan *et al*. 2017a], offering further potential as a viable therapeutic option for individuals with T2DM, since this disorder is characterised by both beta cell loss [Halban *et al*. 2014] and reduced bioactivity of GIP [Vilsboll *et al*. 2002]. However, it should be noted that some have suggested that xenin-25 may inhibit GLP-1 secretions, which if confirmed true, would be detrimental in terms of the antidiabetic actions [Sterl *et al*. 2016], although further research is required to confirm this finding. This initial work, which characterised the impact of xenin-25 on gut motility and energy turnover, together with more recent findings highlighting the encouraging effects on metabolic control, demonstrates favourable and potentially translatable effects.

1.4.2.1 XENIN-25 AS AN ANTIDIABETIC AGENT

Under both normal and type 2 diabetic conditions, numerous studies have demonstrated that xenin-25 can induce the release of insulin and potentiate the biological actions of the incretin hormone GIP [Taylor *et al*. 2010; Wice *et al*. 2010, 2012; Martin *et al*. 2012; Chowdhury *et al*. 2014; Gault *et al*. 2015; Craig *et al*. 2018]. However, the short biological half-life of xenin-25, due to enzymatic degradation, creates a major barrier to its exploitation as a therapeutic agent [Martin *et al*. 2014]. Similarly, for clinical exploitation of GLP-1, enzyme-resistant forms of xenin-25 have

been developed and characterised, and demonstrated clear therapeutic potential (Table 1.3). As such, stabilised forms of xenin-25 have been developed, as outlined below.

1.4.2.2 STABILISED FORMS OF XENIN

Stable, enzyme-resistant forms of xenin-25 have been generated. As such, a fatty acid derivative form of xenin-25, namely xenin-25[Lys^{13}PAL], has been synthesised and fully characterised, with hypothesised antidiabetic effects [Martin *et al* 2012]. A follow-up study, conducted in high fat fed diabetic mice, employing chronic administration of xenin-25[Lys^{13}PAL], confirmed this suggestion, demonstrating augmentation of insulin secretion, enhanced glucose homeostasis and tissue insulin sensitivity, as well as partial restoration of normal islet architecture [Gault *et al*. 2015]. Since the degradation of xenin-25 is primarily due to serine-like proteases [Martin *et al*. 2014; Craig *et al*. 2018], this led to the generation of a modified analogue of xenin-25, where Lysine (Lys) and Arginine (Arg) amino acid residues were substituted for Glutamine (Gln), yielding xenin-25-Gln [Parthsarathy *et al*. 2016]. This novel analogue was enzyme resistant and retained similar biological activity to the parent peptide, xenin-25. Chronic administration of xenin-25-Gln in high fat fed and *ob*/*ob* mice, resulted in enhanced sensitivity to GIP, improved metabolic control and an improved circulating lipid profile [Parthsarathy *et al*. 2016]. These observations with stabilised, potent and long-acting xenin-25 analogues highlight the antidiabetic potential offered by these peptides. However, another approach to help exploitation of this favourable gut hormone could be through increasing xenin concentrations, which could be possibly achieved through MetAP2 inhibitors, which may inhibit xenin degradation, as detailed in section 1.5.

1.4.2.3 XENIN-25 FRAGMENT PEPTIDES

Stable xenin-25 analogues, outlined above, have demonstrated beneficial effects in preclinical models of diabetes-obesity. However, therapeutic attractiveness of these stabilised forms could be further enhanced with truncated and bioactive peptide fragments, which could make peptide synthesis easier and cheaper, as well as facilitating possible non-injectable peptide drug delivery [Novakovic *et al*. 2013; Park *et al*. 2015]. A comprehensive study revealed the degradation profile of xenin-25 in mouse plasma, including evidence of the following C-terminally truncated metabolites; xenin 9-25, xenin 11-25, xenin 14-25 and xenin 18-25, where xenin 18-25 represents xenin-8 (Table 1.3) [Martin *et al*. 2014]. Of these discovered xenin-25 metabolites, only xenin-8 was shown to possess biological activity [Martin *et al*. 2014]. This C-terminal octapeptide of xenin-25 has long been recognised as a naturally occurring bioactive form of xenin-25 [Feurle *et al*. 1997], with studies highlighting the clear effects of xenin-8 on pancreatic endocrine cells [Rodrıguez-Gallardo *et al*. 2001; Silvestre *et al*. 2003], including potent release of insulin [Martin *et al*. 2014]. However, unlike xenin-25 which has effects on satiety, xenin-8 has been shown to have no effect on satiety [Martin *et al*. 2014], which may suggest a change in passage through the blood-brain barrier of this fragment peptide compared to the parent molecule [Kim and Mizuno, 2010]. Interestingly, the C-terminal octapeptide of xenin-25-Gln, namely xenin-8-Gln [Parthsarathy *et al*. 2016], has been shown to retain full gluco-regulatory and antidiabetic actions of the full-length stable analogue [Martin *et al*. 2016]. Additionally, xenin-6 (xenin 20-25) has also underwent characterisation as a biologically active fragment peptide of xenin-25 [Feurle *et al*. 2003], and possesses prominent insulinotropic actions. These notable bioactive actions have been shown to be further enhanced through the introduction of a reduced pseudopeptide bond

between the Lys^{20} and Arg^{21} amino acid residues in xenin-6 [Feurle *et al* 2002; Craig *et al*. 2019]. However, full characterisation of this reduced pseudopeptide, as well as other xenin-6 fragment peptides, is lacking and thus, analogues of xenin-6 may yet hold promise in the treatment of diabetes.

Table 1.3: Amino acid sequences of xenin-25 as well as its related stable analogues and naturally occurring fragment peptides [Craig *et al*. 2018].

Peptide	**Amino acid sequence**
Xenin-25	H-MET-LEU-THR-LYS-PHE-GLU-THR-LYS-SER-ALA-ARG-VAL-LYS-GLY-LEU-SER-PHE-HIS-PRO-LYS-ARG-PRO-TRP-ILE-LEU-OH
Xenin-25-Gln	H-MET-LEU-THR-LYS-PHE-GLU-THR-LYS-SER-ALA-ARG-VAL-LYS-GLY-LEU-SER-PHE-HIS-PRO-GLN-GLN-PRO-TRP-ILE-LEU-OH
Xenin-25[Lys^{13}PAL]	H-MET-LEU-THR-LYS-PHE-GLU-THR-LYS-SER-ALA-ARG-VAL-LYS(N-ε-(γ-GLU(hexadecanoyl))-GLY-LEU-SER-PHE-HIS-PRO-LYS-ARG-PRO-TRP-ILE-LEU-OH
Xenin 9-25	H-SER-ALA-ARG-VAL-LYS-GLY-LEU-SER-PHE-HIS-PRO-LYS-ARG-PRO-TRP-ILE-LEU-OH
Xenin 11-25	H-ARG-VAL-LYS-GLY-LEU-SER-PHE-HIS-PRO-LYS-ARG-PRO-TRP-ILE-LEU-OH
Xenin 14-25	H-GLY-LEU-SER-PHE-HIS-PRO-LYS-ARG-PRO-TRP-ILE-LEU-OH
Xenin 18–25 (Xenin-8)	H-HIS-PRO-LYS-ARG-PRO-TRP-ILE-LEU-OH
Xenin 18–25 Gln	H-HIS-PRO-GLN-GLN-PRO-TRP-ILE-LEU-OH
Xenin 20-25 (Xenin-6)	H-LYS-ARG-PRO-TRP-ILE-LEU-OH

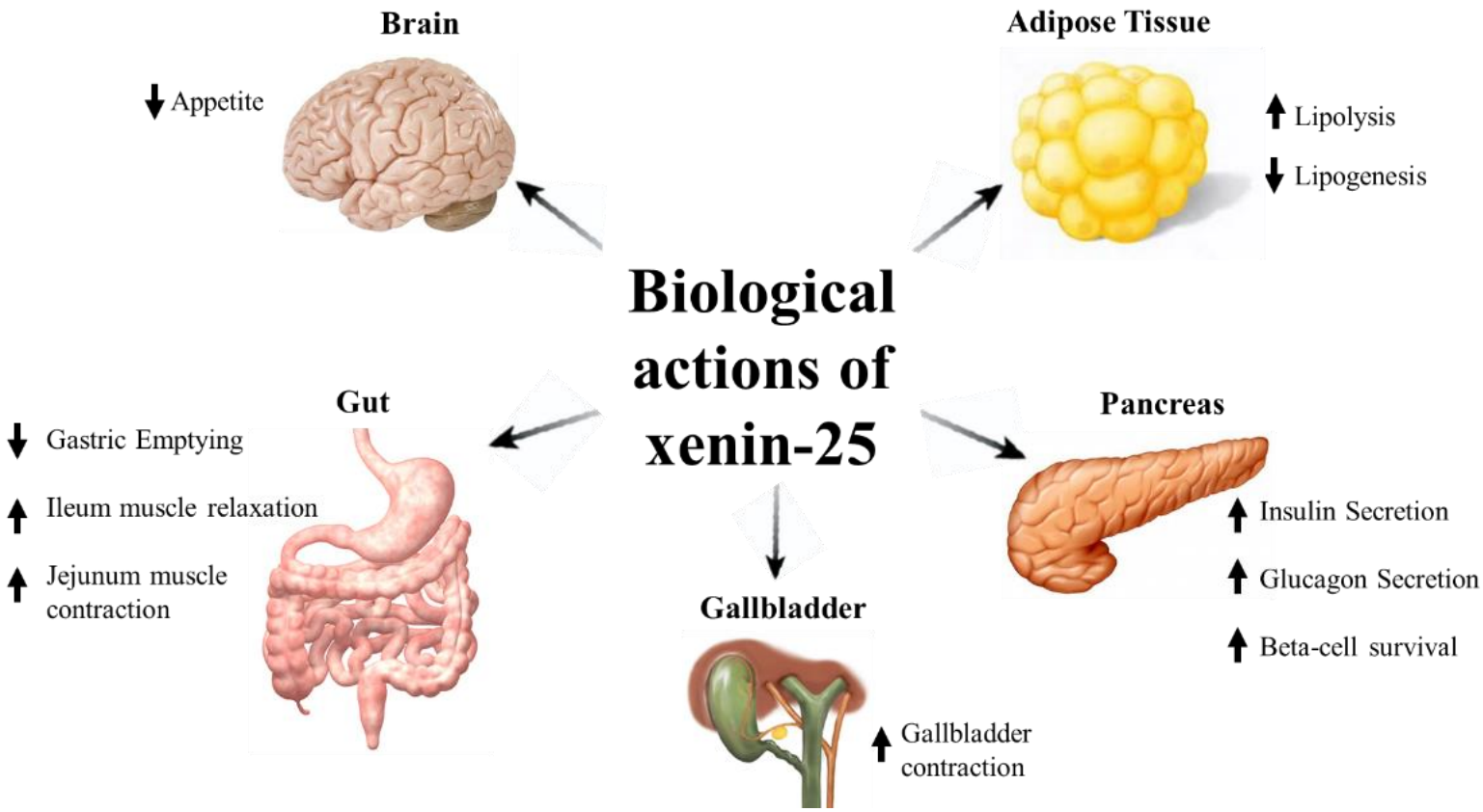


Figure 1.3 Schematic showing the main biological actions of xenin-25 on adipose, brain, gallbladder, gut and pancreas. [Taken from Craig *et al*. 2018].

1.5 TNP-470 – A METHIONINE AMINOPEPTIDASE 2 INHIBITOR

Methionine aminopeptidase 2 (MetAP2) is a bi-functional protein which plays a key role in both the regulation of protein synthesis and post-translational processing. This agent is currently being investigated for potential treatment of metabolic diseases, including obesity and diabetes. MetAP2 is an enzyme responsible for the co-translational cleavage of the N-terminal methionine from nascent proteins, such as in the gut hormone xenin. Therefore, the use of MetAP2 inhibitors may also extend the biological half-life of xenin and allow for clinical exploitation of xenin as a novel therapeutic. By eluding degradation, the favourable effects of xenin, such as inducement of insulin secretion and potentiating the actions of GIP [Craig *et al*. 2017], may be increased within T2DM regimes.

MetAP2 is also known as the molecular target of the angiogenesis inhibitor TNP-470. TNP-470, an analogue of fumagillin, originally shown to be an effective anti-angiogenic agent and an inhibitor of endothelial cell growth [Yeh *et al*. 2006], with efficacy of such inhibitors demonstrated in the treatment of various cancers [Kruger and Figg, 2000; Logothetis *et al*. 2001; Nahari *et al*. 2006]. Studies have also demonstrated their anti-inflammatory, anti-infective and anti-obesity effects [Bråkenhielm *et al*. 2004; Mann-Steinberg and Satchi-Fainaro, 2008]. TNP-470 has also been shown to reduce oxidative stress and hyper-permeability [Mauriz *et al*. 2003; Satchi-Fainaro *et al*. 2005], which are favourable attributes for cancer treatments. The angiostatic mechanism, preventing cell proliferation, includes the suppression of MetAP2 in endothelial cells. Recent studies have also demonstrated the inhibition of proliferation by TNP-470 in fibroblasts [Kria *et al*. 1998], vascular smooth muscle

cells [Ogata *et al*. 2003], hepatic stellate cells [Wang *et al*. 2000] and mesangial cells [Haraguchi *et al*. 1997].

More recent evaluations of this inhibitor have shown and focused on the potential of MetAP2 inhibitors to be used as therapeutic options for the prevention or management of obesity and T2DM, and are already being investigated as a novel drug class [Kim *et al*. 2007; Hughes *et al*. 2013]. Recent studies have confirmed the unique anti-obesity efficacy in various models of obesity / diabetes, as significant weight reductions compared to controls were demonstrated [White *et al*. 2012; Proietto *et al*. 2016]. Significant reductions in food intake was observed, as well as reductions in subcutaneous and omental fat depots. Another study demonstrated that treatment with TNP-470 decreased insulin levels and improved insulin sensitivity [Bråkenhielm *et al*. 2004]. Therefore, this suggests that MetAP2 inhibitors, such as TNP-470, could prove therapeutically favourable as a novel treatment for T2DM due to the relationship between obesity and T2DM, or as a preventative measure. It is suggested that the favourable improvement by MetAP2 inhibitors on glycaemic control and significant weight loss benefits may be through mechanisms involving fat metabolism, storage and fatty acid synthesis [Bråkenhielm *et al*. 2004; Kim *et al*. 2007; Lijnen *et al*. 2010; Elfers and Roth, 2017; Burkey *et al*. 2018]. Additional benefits of TNP-470 in T2DM may include enhancement of xenin levels, with subsequent potentiation of the incretin hormone GIP.

1.6 COMBINATIONAL TREATMENT APPROACHES

Monotherapy frequently fails to elicit all the ideal properties required to successfully treat diseases, therefore low doses of complementary drugs given in combination have

been studied to create a novel treatment paradigm that is closer to an idyllic treatment regimen. Combinational therapeutic approaches could improve patient tolerability, as two different compounds may be employed below their individual side effect dose thresholds. Combinational methods may also offer greater efficacy, with reduced risk and improved adverse effect profile compared to up-titration monotherapy, as well as reduced costs and enhanced drug concordance. Combinational drug approach may also reduce the development of drug resistance.

In T2DM, the majority of patients fail to achieve adequate glycaemic control with a single therapeutic agent [Dodd *et al*. 2009; Bailey *et al.* 2016]. Therefore, the administration of multiple therapeutic agents is required to successfully manage this disorder, and ideally improve clinical outcomes and quality of life for individuals with T2DM [Bell, 2013]. Due to the complex pathogenesis of T2DM, and the multiple metabolic defects associated with T2DM, simultaneously targeting multiple metabolic pathways can improve diabetic status, as single agents currently do not adequately address the complexity of this disease. Metformin is the most common first-line therapy for T2DM, therefore initial combinational approaches included metformin. Metformin has shown success when given in combination with either sulfonylureas, thiazolidinediones, DPP-4 inhibitors, SGLT2 inhibitors, GLP-1 receptor agonists, or with insulin, with triple therapy used when dual combination fails to elicit the set target response. Combination drug choice is based on various characteristics such as the individual patient and disease progression as well as drug efficacy, hypoglycaemic risk, weight loss effects, potential side effects and overall cost. The goal of combination therapy in T2DM is to achieve optimum blood glucose levels whilst minimising potential side effects such as hypoglycaemia.

1.7 HYBRID PEPTIDES

Due to the ever-increasing prevalence of T2DM and obesity, novel treatment options are necessary to help achieve efficient glycaemic control and enable weight loss, as current treatment options contribute to further deterioration due to their weight neutral or gain effects. The alleviation, post bariatric surgery, of diabetes and obesity emphasise the therapeutic importance of the gut-brain axis, and involvement of several gut hormones, therefore necessitating the development of novel patient friendly therapeutic approaches, which can replicate the favourable effects of bariatric surgeries. As stated previously, gastric bypass surgery has been shown to significantly reduce body weight and improve glycaemic control, with complete eradication of T2DM shown because of this surgery [Pournaras *et al*. 2010]. Thus, hybrid agents, entailing gut hormones, could closely mimic the hormonal changes after this surgery, since the effects elicited by gut hormones are multifactorial. Therefore, since surgery is not a viable option for many patients, development of such treatments could potentially provide similar outcomes, but to a larger patient cohort.

Hybrid peptides have the capacity to combine the beneficial effects of multiple compounds, and modulate the various biological pathways involved, within a single compound. A proof of concept for utilisation of hybrid peptides includes the naturally occurring dual agonist oxyntomodulin (OXM), which activates both glucagon-like peptide-1 receptor and glucagon receptor pathways [Pocai, 2013]. These hybrid therapeutics focus on improving patient convenience and compliance, through strategies that allow less-frequent dosing, with possible oral administrations. Chemical approaches for the development of such multifunctional compounds can include a hybrid of two molecules being joined together, either directly or through a linker, or

as chimeras, where the second pharmacological activity is 'designed in' to an already existing peptide backbone [Fosgerau and Hoffmann, 2015]. Hybrid drugs offer various advantages including improved dosage compliance, with reduced toxicity risks, the ability to design better drug combinations using the bioactive regions of peptides, as well as delaying or avoiding the development of drug resistance.

Recent studies have highlighted the possibility of linking together different bioactive peptide fragment domains, to create multi-targeting hybrid peptides [Irwin *et al*. 2015; Mansur *et al*. 2016; Tschöp and DiMarchi, 2017], which stimulates multiple pathways through a single agent. In T1DM, the clinical success of combined modulation through a single drug formulation of insulin and GLP-1 (IDegLira), further highlights the benefits of stimulating multiple signalling pathways for enhanced therapeutic outcomes [Kapitza *et al*. 2015]. In T2DM, Gault *et al*. 2011 demonstrated improved glucose-lowering and insulinotropic properties from a GLP-1 and GIP combination. Furthermore, a CCK/GLP-1 hybrid has been characterised, displaying notable benefits on appetite, insulinotropic effects as well as pancreatic beta cell function and morphology [Irwin *et al*. 2015]. Hasib *et al*. (2017) demonstrated the promising antidiabetic properties of a GIP/xenin hybrid, namely ($DAla^2$)GIP/xenin-8-Gln. Other hybrid peptides displaying therapeutic efficacy for the treatment of diabetes / obesity includes a GIP-oxyntomodulin hybrid, showing weight reducing and favourable anti-diabetic effects [Bhat *et al*. 2013] and triple acting hybrid peptides incorporating GIP/glucagon/GLP-1, displaying therapeutic potential in high fat fed mice [Gault *et al*. 2013]. Given the initial promise of the above agents, and considering GIP and xenin are both secreted from the same intestinal K-cells, it would seem reasonable to assume that combining GIP with xenin in a hybrid agent could be favourable for the treatment

of T2DM. Additionally, given the evidence against augmenting GIP in T2DM treatment [Gault *et al*. 2003b; Irwin *et al*. 2004, 2019], employing hybrid agents may improve efficacy, compared to monotherapy. Dual or triple target pharmacology, such as the aforementioned multifunctional hybrid peptides, as opposed to single target pharmacology, could present new therapeutic challenges due to the complexity in targeting various receptors and signalling pathways, especially in translation from animal to human situations. However, it is highly probable that such hybrid agents will offer superior efficacy, together with reduced adverse effects and low dose administration [Irwin and Flatt, 2015], as well as being more patient friendly.

1.8 AIMS OF THESIS

The primary aim of this thesis is to capitalise on the previous success of xenin-25 analogues, as well as related hybrid peptides, to further build on prospects of re-sensitising the actions of the incretin hormone GIP, and to establish their insulinotropic, glucose-lowering and satiety effects in different mouse models of obesity and diabetes.

Main aims and objectives:

1. To assess the chronic and persistent in vivo antidiabetic benefits of (DAla^2)GIP/xenin-8-Gln, in combination with the clinically approved GLP-1 mimetic exendin-4, and examine its persistent beneficial metabolic effects in high fat fed mice.

2. To investigate the *in vitro* and acute *in vivo* biological actions and potential antidiabetic properties, including glucose-lowering, insulinotropic and satiety effects, of a series of modified novel xenin-6 peptides.

3. To evaluate the antidiabetic therapeutic efficacy of Ψ-xenin-6 and sitagliptin, alone or in combination, in STZ treated high fat fed mice.

4. To examine the antidiabetic and anti-obesity properties of 18-day combined administration of TNP-470 and sitagliptin on anti-hyperglycaemic, insulinotropic and anti-obesity activity in STZ treated high fat fed mice.

Chapter 2

General Materials and Methods

2.1 MATERIALS

Purified water was obtained from an Elga PURELAB Ultra system (Elga, Celbridge, Ireland). Chemicals and reagents were sourced as follows:

Abcam (Cambridge, UK): mouse monoclonal antibody to insulin, rabbit monoclonal antibody to glucagon and rabbit polyclonal antibody to ki-67

BDH Chemicals Ltd. (Poole, UK): Calcium chloride ($CaCl_2.6H_2O$), dichloromethane (CH_2Cl_2), di-sodium hydrogen orthophosphate (Na_2HPO_4), ethanol, D-glucose, hydrochloric acid (HCl), magnesium sulphate ($MgSO_4.7H_2O$), paraformaldehyde (PFA), potassium dihydrogen orthophosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), sodium dihydrogen orthophosphate (NaH_2PO_4) and tri-sodium citrate ($C_6H_5Na_3O_7.2H_2O$).

Gibco Life Technologies Ltd. (Paisley, UK): RPMI 1640 tissue culture medium, antibiotics (100U/ml penicillin and 0.1g/L streptomycin), Dulbecco's Modified Eagle Medium (DMEM) (25 mmol/l glucose) supplemented with 2 mmol/l L-glutamine, 10% (v/v) FCS and antibiotics (100 U/ml penicillin and 0.1 g/l streptomycin), foetal bovine serum (FBS), Hanks buffered saline solution (HBSS 10x stock) and trypsin/EDTA (10x stock).

Invitrogen (Paisley, UK): Alexa Fluor 594 goat anti-mouse IgG (H+L), Alexa Fluor 488 goat anti-mouse IgG (H+L), Alexa Fluor 594 goat anti-guinea-pig IgG (H+L), Alexa Fluor 488 goat anti-guinea-pig IgG (H+L), Alexa Fluor 594 goat ant-rabbit IgG (H+L), Alexa Fluor 488 goat anti-rabbit IgG (H+L) and xylene.

Millipore (Hertfordshire UK): Rat/mouse GIP (total) ELISA, GLP-1 total ELISA and Glucagon ELISA Kit

Novo Industria (Copenhagen, Denmark): Rat insulin standard

Perkin Elmer (Cambridge, UK): Sodium iodide – $Na^{125}I$ (74Mbq/20µl stock)

Phoenix Pharmaceuticals (California, USA): Xenin 25 ELISA Kit

Rathburn (Walkersburn, Scotland, UK): Dichloromethane (DCM)

Roche Diagnostics (Sussex, UK): LightCycler 480 SYBR Green I Master and LightCycler 480 Multi-well Plates

Sigma-Aldrich Chemical Company Ltd. (Poole, UK): Acetonitrile, α-cyano-4-hydroxycinnamic acid, bovine insulin (crystalline), bovine serum albumin (essentially fatty acid free, endotoxin free), charcoal (activated/untreated), collagenase-V, dextran T-70, 4',6-diamidino-2-phenylindole (DAPI), diethyl pyrocarbonate (DEPC),

dimethyl sulphoxide (DMSO), C,N-diphenyl-N'-(4,5 dimethylthiazol-2-yl) tetrazolium bromide (MTT), ethanol, ethylene diaminetetraacetic acid (EDTA), glycerol, hydrogen peroxide (H2O2; 30% w/w), N-2-hydroxyethylpeprazine N'-2-ethanesulphonic acid (HEPES), isopropanol, 2-mercaptoethanol, palmitic acid sodium salt, Phosphate buffered saline (PBS) tablets, potassium chloride (KCl), sodium hydroxide (NaOH), streptozotocin, sequencing grade trifluoroacetic acid (TFA), 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (iodogen), thimerosal, Triton X-100, trizma base, trizma hydrochloride, trypan blue solution (0.4%), TUNEL reaction mixture and tween-20.

VWR International (Lutterworth, UK): Disodium hydrogen orthophosphate (Na_2HPO_4) and sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) and Polysine^{TM} coated slides.

2.2 PEPTIDE SYNTHESIS

Native xenin-25 was synthesised by GL Biochem (Shanghai, China; >95% purity). Xenin-6, xenin-6-NH_2, xenin-6-Gln and xenin-6-Gln-NH_2 (Table 2.1) were synthesised by Synpeptide Ltd (Shanghai; China; >95% purity). Ψ-xenin-6 was obtained from Saxon Biochemicals (Hannover, Germany). All peptides were procured at >95% purity. Sitagliptin phosphate monohydrate, used in chapters 5 and 6, was purchased from Apex Bio Technology (Houston, TX, USA; >97% purity; MW 523.3; CAS No. 654671-77-9). TNP-470, used in chapter 6, was purchased from Apex Bio Technology (>98% purity; MW 401.9; CAS No. 129298-91-5).

2.3 PEPTIDE PURIFICATION AND CHARACTERISATION

2.3.1 Peptide purification confirmation using reverse phase high performance liquid chromatography (RP-HPLC)

The purity of all peptides, dissolved in distilled water at a concentration of 1 mg/ml, was confirmed using a RP-HPLC column on a Spectra Series P200 chromatography

system attached to Spectra Series UV100 detector. 40 µl of each peptide solution was made up to 100 µl using 0.1% (v/v TFA/water) and injected into HPLC column for succeeding spectra analysis, using 70% acetonitrile as eluting solution. This was chromatographed using Kinetex C-18 analytical column (150 x 4.60 mm, Phenomenex, Chesire, UK) equilibrated with 0.1% (v/v) TFA/water at flow rate of 1.5 ml/min. Using linear gradients, the concentration of eluting solution was raised from 0 to 20% acetonitrile over 2 minutes followed by 20 to 80% over 27 minutes, followed by 80 to 100% over 3 minutes. Absorbance was measured at 214 nm. Collected peaks were analysed using Thermo Electron ChromQuest data collection software (version 3). Purified fractions were further analysed by mass spectrometry (Section 2.3.2).

2.3.2 Confirmation of molecular mass by matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)

The molecular weight of each peptide was confirmed using a Voyager-DE Biospectrometer (PerSeptive Biosystems, Hertfordshire, UK), equipped with a 1 metre time-of-flight tube. Matrix solution consisted of 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid (CHCA), prepared in 80% acetonitrile, 20% water with 0.1% TFA. 10 µl of each peptide sample was mixed with 10 µl of matrix solution and applied to a predefined well on a 100-well stainless steel plate and allowed to dry at room temperature. Each sample measured was collected in linear positive ionisation mode using 100 laser shots/spectrum. Mass spectra were recorded as mass-to-charge (m/z) ratio against relative abundance. Experimental masses obtained for each peptide were compared to theoretical masses [Gault *et al.* 2003a].

2.4 METABOLIC STABILITY STUDIES

2.4.1 Assessment of plasma degradation of peptides

Murine plasma was collected as described in Section 2.15. Plasma degradation of peptides was performed as follows; 40 µl of peptide (1 mg/ml) was incubated at 37 ^{o}C, with 10 µl of murine plasma in 380 µl triethanolamine-HCl (50 mmol/l, pH 7.8) for 0, 120, 240 and 360 minutes. Reactions were stopped via addition of 50 µl of 10% (v/v) TFA/water. Degradation products were separated using HPLC (Section 2.3.1), with MALDI-TOF MS analyses of collected peaks (Section 2.3.2).

2.5 CELL CULTURE

Derived from electrofusion of New England Deaconess Hospital (NEDH) rat islets and immortal rat insulinoma RINm5F cells [McClenaghan *et al.* 1996], BRIN-BD11 cells were cultured in 25 ml pre-warmed RPMI-1640 growth media (11.1 mmol/l glucose), supplemented with 10% v/v FBS and 1% v/v antibiotics (0.1 mg/ml streptomycin and 100 U/ml penicillin) in 75 cm^2 vented sterile tissue culture flasks (Nunc, Roskilde, Denmark). Cells were maintained at 37 ^{o}C and 5% atmospheric CO_2, in LEEC incubator (Laboratory technical engineering, Nottingham, UK) as described previously by McClenaghan *et al.* [1996]. INS-1 cells have a high insulin content and are a glucose responsive cell line. The INS-1 cell line was cultured in RPMI-1640 (11.1 mol/l glucose), supplemented with 10% FBS, 1% penicillin-streptomycin (5000 U/l), 200 µM 2-mercatoethanol and 1 mmol/l sodium pyruvate. The hybrid cell line 1.1B4 is derived from electrofusion of a primary culture of human pancreatic islets with PANC-1, a human pancreatic ductal carcinoma cell line [McCluskey *et al*. 2011]. Cells were cultured at 37 ^{o}C and 5% CO_2 in RPMI-1640 media containing 11.1 mmol/l glucose and 2 mmol/l L-glutamine supplemented with 10% (v/v) foetal calf serum

(FCS) and antibiotics (100 U/ml penicillin and 0.1 g/l streptomycin). α-TC1.9 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mmol/l glucose and 2 mmol/l L-glutamine supplemented with 10% (v/v) FCS and antibiotics (100 U/ml penicillin and 0.1 g/l streptomycin).

Prior to experimentation, all cells were routinely passaged in the following manner. Once 70-80% confluent, culture media was decanted from flasks and cells washed with 10 ml pre-warmed HBSS. After washing, cells were incubated at 37 ºC for 3-5 minutes with 3 ml pre-warmed dissociation agent, trypsin/EDTA (0.025%, w/v), to allow cell detachment. Action of trypsin was stopped by addition of fresh culture media (7 ml), and pipetted, forming a single suspension, which was then transferred to a 50 ml Sterilin tube (Sterilin Ltd., Hounslow, UK). This was centrifuged for 5 minutes at 900 rpm. The supernatant was discarded and the pellet was re-suspended in a known volume of fresh tissue culture medium. For maintenance cell cultures, cells were re-suspended in fresh tissue culture medium (25 ml) and maintained at 37 ºC until 70-80% confluent. For experimentation assays, the number of cells were determined using a Neubauer haemocytometer (Scientific Supplies Co., UK), after staining 100 µl of cell suspension with 100 µl trypan blue. Viable cells were counted in all four WBC squares and averaged to obtain the total number of cells in suspension.

2.6 INSULIN SECRETION FROM BRIN-BD11 AND INS-1 CELLS

2.6.1 Acute *in vitro* insulin release studies from BRIN-BD11 and INS-1 cells

BRIN-BD11 and INS-1 cells were used to assess insulin releasing activity of all peptides. Cells used for experiments were between passages 16 and 35. Acute tests for insulin release were performed according to established laboratory protocol. Cells

were harvested and counted as described in Section 2.5, and seeded at density of 150,000 cells per well in 24-well plates (Flacon Ltd) and supplemented with 1 ml of RPM1-1640 media. Cells were allowed to attach overnight at 37^{o}C to form monolayers. Following attachment, culture media was decanted and cells pre-incubated in 1 ml Krebs-Ringer bicarbonate buffer (KRBB- 115 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.28 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 25 mmol/l HEPES and 8.4% $NaHCO_3$, containing 0.5% (w/v) BSA, pH 7.4) supplemented with 1.1 mmol/l glucose for 40 minutes at 37 ^{o}C. Following pre-incubation, the solution was removed and fresh buffer containing either 1.1, 5.6 or 16.7 mmol/l glucose (BRIN-BD11 cells), or 5.6 mmol/l glucose (INS-1 cells) along with a known concentration of peptide (10^{-12} to 10^{-6} mol/l) was added to each well and incubated for 20 minutes at 37 ^{o}C. Following test incubation, 950 µl assay buffer from each well was collected and duplicate aliquots of 200 µl stored at -20^{o}C for measurement of insulin by radioimmunoassay (RIA) as outlined in Section 2.11.2. In a separate set of experiments, BRIN-BD11 cells were incubated, as above, at 5.6 mmol/l glucose with test peptides in the presence of GIP (10^{-6} mol/l), and insulin measured by RIA as described in Section 2.11.2. In another set of experiments, BRIN-BD11 cells were incubated, as above, in glucotoxic conditions (22.2 mmol/l glucose) with test peptides alone (10^{-6} mol/l), or in the presence of GIP (10^{-6} mol/l), and insulin measured by RIA.

2.7 DETERMINATION OF PEPTIDE TOXICTY

2.7.1 Cytotoxicity assay

Lactate dehydrogenase (LDH) is released from cells when their membrane integrity has been lost. In this study, toxicity of test peptides (10^{-6} mol/l, 20 minutes) was determined by assessing levels of LDH released in BRIN-BD11 cells, with dimethyl

sulfoxide (DMSO, Sigma-Aldrich, UK) used as a positive control. This was performed using the cell supernatants obtained from the acute insulin-release experiments. Concentrations of LDH were measured using a CytoTox96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI) according to manufacturer's protocol. This is a colorimetric method of assessing the number of nonviable cells within a cell population. Following insulin release studies, 50 µl aliquots of the previously obtained supernatant was mixed with an equal volume of LDH assay buffer in 96-well microtiter plates (n=4; Orange Scientific, California, USA). Due to the light sensitive nature of the LDH assay buffer, samples were incubated and covered in foil for 30 minutes at room temperature. To stop the reaction, 50 µl of stop solution was added to samples. Samples were then measured using SoftMaxPro software on FlexStation fluorimeter (Molecular devices, Sunnyvale, USA), with LDH levels detected at an absorbance of 490 nm as described by Allen *et al.* [1994] and Srinivasan *et al*. [2014]. This assay is highly sensitive and capable of accurately measuring the lower levels of LDH known to be present within beta cells [Schuit *et al.* 2001].

2.8 GLUCAGON SECRETION IN α-TC1.9 CELLS

2.8.1 Acute glucagon secretion from α-TC1.9 cells

α-TC1.9 cells were harvested and counted as described above (Section 2.5). Cells were seeded and cultured for 48-72 h at 37 ^{o}C in 24-well plates at a cell density of 100,000 cells per well in 1 ml DMEM containing 25 mmol/l glucose and 2 mmol/l L-glutamine supplemented with 10% (v/v) FCS and antibiotics (100 U/ml penicillin and 0.1 g/l streptomycin) culture medium to ensure attachment. Before commencement of acute studies, cells were pre-incubated with 1 ml KRBB supplemented with 20 mmol/l glucose for 1 h at 37 ^{o}C. Following removal of pre-incubation buffer, cells were

incubated for 2 h at 37 ^{o}C with glucose (1.4 mmol/l) and test peptides (10^{-6} mol/l) alone or in combination with GIP (10^{-6} mol/l). Following test incubation, 900 µl assay supernatant from each well was removed and stored at -20 ^{o}C until glucagon measurement by chemiluminescent enzyme-linked immunosorbent assay (ELISA; Millipore, Watford, UK) as outlined in Section 2.12.1.

2.9 PROLIFERATION AND APOPTOSIS

2.9.1 *In vitro* beta-cell proliferation and apoptosis

Rodent BRIN-BD11 and human 1.1B4 beta-cells were used to investigate effects of test peptides on beta-cell proliferation and protection against cytokine-induced apoptosis. GLP-1 (10^{-8} and 10^{-6} mol/l) was employed as a positive control for all studies. Ki-67 immunostaining was used to assess effects on proliferation. Cells were cultured as previously described above (Section 2.5), and seeded onto coverslips at a cell density of 40,000 cells per coverslip and cultured overnight (18 h) at 37 °C, in the presence of test peptides (10^{-8} and 10^{-6} mol/l). Cells were then washed with PBS, and fixed using 4% paraformaldehyde. Following antigen retrieval with citrate buffer at 90 °C for 20 minutes, cells were blocked using 1.1% BSA for 30 minutes. Cells were then incubated with Ki-67 primary antibody (1:200; Abcam ab16667), followed by Alexa Fluor 594 goat anti-rabbit IgG (1:400). Coverslips were washed with PBS, mounted on slides for viewing using a FITC (488 nm) or TRITC filter (594 nm) fluorescent microscope (Olympus System Microscope) and photographed by DP70 camera adapter system. Proliferation frequency was expressed as percentage of total cells analysed as detailed in Section 2.17.

For analysis of the ability of test peptides to protect against cytokine-induced apoptosis, cells were seeded as detailed above (Section 2.5). However, cells were also

exposed to a cytokine-cocktail (IL-1β 300 U/ml, IFN-γ 300 U/ml, TNF-α 40 U/ml; [Vasu *et al.* 2014a]) in the presence or absence of test peptides (10^{-8} and 10^{-6} mol/l) for 2 h, with hydrogen peroxide as an additional control. TUNEL assay was performed using fluorescein in situ cell death detection kit, according to the manufacturer's protocol (Roche Diagnostics, UK). In brief, following overnight incubation, cells were washed with PBS and fixed using 4% paraformaldehyde at room temperature for 30 minutes. Following another wash, cells were incubated in sodium citrate buffer (94 ^{o}C for 20 minutes; 10 mmol/l Sodium Citrate, 0.05% Tween 20, pH 6.0), and then allowed to cool (20 minutes). Cells were incubated in TUNEL solution for 1 h at 37 ^{o}C before subsequent rinsing in PBS and mounted onto slides for viewing under a fluorescent microscope (Olympus System Microscope) as above. Apoptosis was expressed as percentage of total cells analysed. Approximately 150 cells were analysed per group.

2.10 INSULIN AND GLUCAGON SECRETION FROM ISOLATED MOUSE ISLETS

2.10.1 Islet isolation by collagenase digestion

Described previously by Lacy and Kostinosky [1967], islets from mouse (NIH Swiss mice, male, 10-12 weeks) pancreas were isolated using a modified collagenase digestion method [Gunawardana *et al.* 2004]. Stock islet isolation HBSS was prepared by dissolving 136.9 mmol/l NaCl, 5.4 mmol/l KCl, 1.3 mmol/l $CaCl_2$, 0.4 mmol/l $MgSO_4.7H_2O$, 0.5 mmol/l $MgCl_2.6\ H_2O$, 0.4 mmol/l $Na_2HPO_4.H_2O$, 0.4 mmol/l KH_2PO_4, 5.6 mmol/l glucose, 0.06 mmol/l phenol red and 4.2 mmol/l $NaHCO_3$ in distilled water. Collagenase solution (5 ml per pancreas) was prepared by adding 1.4 mg/ml collagenase to stock islet isolation HBSS. Wash buffer was prepared by adding 0.1% BSA to stock islet isolation HBSS on the day of islet isolation. Mice were culled

by cervical dislocation and pancreata removed and placed on ice-cold collagenase solution and cut with scissors to aid digestion. Pancreas collagenase mix was placed in a shaking water bath for 10 minutes at 37 ^{o}C to allow digestion. The tube was removed and shaken vigorously for a few seconds to dissociate islets and separate exocrine tissues. The tubes were then filled with ice-cold wash buffer and centrifuged at 1200 rpm for 2 minutes. After centrifugation, supernatant was poured off. This washing procedure was repeated three times and unwanted tissues were removed by filtration. Filtrate was centrifuged for 2 minutes at 1200 rpm and supernatant discarded. The pellet obtained was re-suspended in pre-warmed RPMI-1640 media supplemented with 10% BSA, 1% penicillin/streptomycin and transferred to petri-dishes. Islets were cultured at 37 °C and 5% CO_2, in a LEEC incubator for 2-3 days prior to use.

2.10.2 Acute insulin release studies from isolated mouse islets

Acute insulin release on isolated islets was performed as described previously by Pathak *et al*. (2015). Following 48 h culture, 10 islets were picked under the microscope into 1.5 ml Eppendorf tubes. Tubes were centrifuged at 2000 rpm for 1 minute to remove excess media. Islets were pre-incubated in 1 ml of Krebs–Ringer bicarbonate buffer (KRBB- 115 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.28 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 25 mmol/l HEPES and 8.4% $NaHCO_3$, containing 0.5% (w/v) BSA, pH 7.4) supplemented with 1.4 mmol/l glucose for 1 h at 37 ^{o}C. The pre-incubation buffer was decanted after centrifugation at 2000 rpm for 1 minute. Test incubations were then carried out for 1 h at 16.7 mmol/l glucose with a range of concentrations of peptides (10^{-8} to 10^{-6} mol/l). After incubation, tubes were centrifuged at 2000 rpm for 1 minute and 950 µl supernatant from each tube was

collected. Duplicate aliquots of 200 µl were stored at -20 ^{o}C for measurement of insulin by dextran-coated charcoal radioimmunoassay (Section 2.11.2). Acid ethanol extraction for total islet insulin content was carried out. Briefly, after retrieval of test solutions, 500 µl of ice-cold acid ethanol solution (1.5% HCl, 75% ethanol and 23.5% H_2O) was added to each Eppendorf tube containing islets and incubated overnight at 4 ^{o}C. Supernatant was collected after centrifugation and stored at -20 ^{o}C. For measurement of total insulin content by RIA (Section 2.11.2), 5 µl of test sample was diluted with 195 µl of working RIA buffer (1:40 dilution).

2.10.3 Acute glucagon release studies from isolated mouse islets

Islets were isolated as above (Section 2.10.1). Following 48 h culture, 10 islets were picked under the microscope into 1.5 ml Eppendorf tubes. Tubes were centrifuged at 2000 rpm for 1 minute to remove excess media. Islets were pre-incubated in 1 ml of Krebs–Ringer bicarbonate buffer (KRBB- 115 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.28 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 25 mmol/l HEPES and 8.4% $NaHCO_3$, containing 0.5% (w/v) BSA, pH 7.4) supplemented with 1.1 mmol/l glucose for 1 h at 37 ^{o}C. The pre-incubation buffer was decanted after centrifugation at 2000 rpm for 1 minute. Test incubations were then carried out for 1 h at 1.4 mmol/l glucose with test peptides (10^{-6} mol/l) alone or in combination with GIP (10^{-6} mol/l). After incubation, tubes were centrifuged at 2000 rpm for 1 minute and 950 µl supernatant from each tube was collected. Duplicate aliquots of 200 µl were stored at -20 ^{o}C for measurement of glucagon secretion using a glucagon chemiluminescent ELISA kit (Section 2.12.1). For total islet glucagon content, acid ethanol extraction was carried out. Briefly, after retrieval of test supernatant above, 500 µl of ice-cold acid ethanol solution (1.5% HCl, 75% ethanol and 23.5% H_2O) was added to each Eppendorf tube

containing islets and incubated overnight at 4 ^{o}C. Supernatant was collected after centrifugation and stored at -20 ^{o}C until glucagon chemiluminescent ELISA (Section 2.12.1).

2.11 INSULIN RADIOIMMUNOASSAY

2.11.1 Preparation of iodinated bovine insulin for RIA

Iodinated bovine insulin was prepared as per the established protocol within the Diabetes Research Group. Iodogen solution (100 μg/ml; 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) was prepared in dichloromethane, with 100 μl of iodogen solution aliquoted into a series of Eppendorf's. Following overnight solvent evaporation in a fume hood, a uniform coating of iodogen was attained at the bottom of each Eppendorf tube. This uniform coating acted as a catalyst during the iodination process of bovine insulin. 1 mg of bovine insulin was dissolved in 1 ml of 10 mmol/l HCL, and diluted to 125 μg/ml (1:8 dilution) in 500 mmol/l phosphate buffer (pH 7.4). Bovine insulin (20 μl) along with sodium iodide (5 μl; $Na^{125}I$ 100 mCi/ml stock, Perkin Elmer, Cambridge, UK) were added to the iodogen-coated tube. This reaction was placed on ice for 15 minutes, with gentle agitation every 3-4 minutes, with the reaction being stopped by transferring the reaction mixture into a clean Eppendorf tube. The reaction tube was then washed with 500 μl of 50 mmol/l sodium phosphate buffer, with this being combined with the reaction mixture in the fresh Eppendorf tube.

Using RP-HPLC, the iodinate mixture was separated using a Vydac C-8 (250 x 4.6 mm) analytical column at flow rate of 1.0 ml/minute with 0.12% (v/v) TFA/water. The concentration of acetonitrile in eluting solution was increased from 0% to 56% over 60 minutes and then to 70% in 5 minutes. Fractions were collected every minute using

a fraction collector (Frac-100, LKB) during the 67-minute HPLC run. Each collected fraction (5 µl) was aliquoted into LP3 tubes and counts measured on a gamma counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter). Fractions with highest counts were selected and double diluted with 1 ml of 40 mmol/l sodium phosphate buffer (pH 7.4) containing 1 g/100 ml BSA and 0.02 g/100 ml thimerosal. Binding capacities of selected fractions were tested with different antibody dilutions (1:25,000-1: 45,000). Fractions having similar binding affinities were pooled together and kept at 4ºC until use in RIA.

2.11.2 Modified dextran-coated charcoal RIA

Using guinea pig anti-porcine antibody, rat insulin standard and ^{125}I-labelled bovine insulin, the amount of insulin release was measured using a modified dextran-coated charcoal radioimmunoassay, as described by Flatt and Bailey (1982). Stock RIA buffer was prepared by adjusting the pH of a basic solution of 40 mmol/l disodium hydrogen orthophosphate (containing 0.3% (w/v) sodium chloride and 0.02% (w/v) thimerosal) to 7.4 with an acidic solution of 40 mmol/l sodium dihydrogen orthophosphate. Stock RIA buffer was kept at 4°C until required. On the first day of RIA, working RIA buffer was prepared by dissolving 0.5% (w/v) BSA in 40 mmol/l sodium phosphate buffer (pH 7.4). Unknown samples were aliquoted in duplicate (200 µl) into LP3 tubes as outlined previously in Section 2.6.1. Insulin standards (0.039 to 20 ng/ml) were prepared through serial dilution of frozen stock rat insulin standard 40 ng/tube (100 µl) with working RIA buffer. To obtain 40% binding, guinea pig anti-porcine antibody was diluted (1:25000 to 1:45000) from frozen stock in working RIA buffer. 100 µl of diluted antibody was then added to 200 µl of standards (in triplicates) and unknown samples (in duplicates). A total counts tube (no reagents), a non-specific

binding tube (only assay buffer) and a control tube (antibody plus assay buffer) were also set up in triplicates. ^{125}I-labeled tracer was added to working RIA buffer to achieve 10,000 counts/100 µl of solution. 100 µl of tracer was then added to each LP3 tube and incubated for 48 h at 4ºC. To separate bound and unbound ^{125}I-labelled insulin, activated charcoal was added following the 48 h incubation. Charcoal solution (5%) coated with dextran T-70 was diluted with stock RIA buffer (1:5 dilution) and 1.0 ml added to each tube (except the total tubes) and incubated at 4ºC for 20 minutes. Following centrifugation at 2500 rpm for 20 minutes at 4ºC in a Sorvall centrifuge, supernatant was poured off and radioactivity of the unbound (free) ^{125}I-labelled insulin remaining absorbed to black charcoal pellets was determined using a gamma counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter). Counts bound to antibody (total counts minus counts bound to charcoal) were inversely proportional to the amount of insulin present in standards or unknown samples.

2.12 GLUCAGON CHEMILUMINESCENT ELISA

2.12.1: Measurement of glucagon secretion

A glucagon ELISA kit was used to quantitatively determine intact glucagon in cell culture supernatants following acute peptide administration. This assay is based on a sandwich ELISA for the capture of glucagon molecules measured by a chemiluminescent substrate. The enzymatic activity was measured by relative light units (RLU) which was proportional to amount of captured glucagon in the unknown sample. For sample preparation, 150 µl of cell supernatant was added to 225 µl of acetonitrile, vortexed and incubated at room temperature for 10-30 minutes. Samples were then centrifuged at 13,000 rpm for 5 minutes using a Beckman microcentrifuge (Beckman Instruments, UK). Supernatant (300 µl) was aspirated to a fresh tube and

concentrated to dryness using an AES1000 Speed-Vac concentrator (Life Sciences International, Runcorn, UK) and stored at 4 ^{o}C.

All contents of the kit were allowed to reach room temperature prior to use and the assay was performed as outlined by the manufacturer's protocol. A glucagon standard curve ranging from 0 – 2 ng/ml was prepared by dilution of the 2 ng/ml stock. Quality controls 1 and 2 were reconstituted in distilled water. All unknown samples were rehydrated with 60 µl assay buffer and the 96 well assay plate was washed with 300 µl diluted wash buffer. Assay buffer was added to blank wells (30 µl) and 20 µl to all other wells. Glucagon standards and quality controls (10 µl) were added in duplicate and unknown samples (10 µl) were added in triplicate to corresponding wells. Antibody mixture solution (20 µl) was then added to each well and incubated for 44-48 h at 4 ^{o}C and 500 rpm. Following incubation period, solutions were decanted and each well was washed thrice with diluted wash buffer (300 µl). Enzyme solution (100 µl) was added to each well and incubated for 30 minutes at room temperature. The 96 well assay plate was washed six times with 300 µl diluted wash buffer. Substrate solution (100 µl) was added to each well, and RLU was measured at 425 nm within 5 minutes of adding substrate. The amount of glucagon in the sample was estimated using reference curve of known glucagon standards (ranging from 0 ng/ml – 2 ng/ml).

2.13 ANIMAL MODELS

In vivo studies in this thesis were carried out using several animal models of obesity, diabetes and insulin resistance. Animal experiments were carried out in accordance with UK Animals (Scientific Procedures) Act 1986 and approved by the University of Ulster Animal Welfare and Ethical Review Body (AWERB). All mice were housed

individually in temperature-controlled rooms (22 ± 2 °C) with a 12 h light:12 h dark cycle, at Ulster University's Behavioural and Biomedical Research Unit (BBRU). No adverse effects were observed after drug treatment. When conducting studies, all animals had free access to food and drinking water, unless stated.

2.13.1 NIH Swiss mice

NIH Swiss mice (male, 10-12 weeks, n=8) were obtained from Envigo, Huntingdon, UK. All animals were age matched, had free access to drinking water and standard laboratory chow (10% fat, 30% protein, 60% carbohydrate; percentage of total energy 12.99 KJ/g; Trouw Nutrition, Cheshire, UK). Groups of mice were used to conduct acute animal studies and used as model of normal glycaemia in chronic studies.

2.13.2 High fat fed NIH Swiss mice

This is a robust model for early T2DM, and is widely used in studies relating to testing new therapeutic methods [Winzell and Ahrén, 2004]. In chapter 3, this model was used for sub-chronic animal studies, with all mice maintained on a high-fat diet (45% fat, 35% carbohydrate and 20% protein; percentage of total energy 26.15 kJ/g; Special Diet Services, UK) for 3 months prior to commencement of treatment. The high fat fed mice resulted in progressive body weight gain (45.8 ± 0.9 *vs.* 35.0 ± 1.2 g; $P<0.001$), hyperglycaemia (14.6 ± 1.8 *vs.* 8.2 ± 0.2 mmol/l; $P<0.05$) and hyperinsulinemia compared with aged matched controls fed on normal laboratory chow. Prior to commencement of the long term treatment regimen, mice were grouped according to body weight and non-fasting blood glucose.

2.13.3 Streptozotocin treated high fat fed C57BL/6 mice

This animal model of T2DM induces insulin resistance and beta cell dysfunction through combination of high fat diet and low dose streptozotocin (STZ) [Zhang *et al*. 2008; Gilbert *et al*. 2011]. Moreover, it is an animal model of T2DM that resembles the human disease [Vatandoust *et al.* 2018]. In chapter 5, before commencing studies, male C57BL/6 mice were given free access to high fat diet. Following two weeks of high fat feeding, mice received a low dose STZ injection (50 mg/kg bw, i.p., in sodium citrate buffer, pH 4.5), followed by another low dose STZ injection (75 mg/kg bw,) one week later. Mice were fasted for 18 h before each STZ injection, with fresh STZ-sodium citrate buffer solution prepared each time. In this study, blood glucose was monitored at regular intervals and animals displayed progressive hyperglycaemia (8.7 ± 0.3 *vs.* 16.8 ± 2.3 mmol/l; $P<0.01$) compared with age-matched controls on normal laboratory chow. Mice exhibiting blood glucose concentrations ≥ 11.1 mmol/l at the end of the 4-week period were then recruited into the study.

In chapter 6, male C57BL/6 mice were given access to high fat diet. Following two weeks of high fat feeding, mice received one low dose STZ injection (50 mg/kg), followed by two low dose STZ injection (75 mg/kg) on weeks 3 and 4. Mice were fasted for 18h before each STZ injection. Mice exhibiting blood glucose concentrations ≥ 11.1 mmol/l at the end of 5-week period were recruited into the study.

2.14 ACUTE ANIMAL STUDIES

2.14.1 Acute food intake studies

Acute cumulative food intake studies were carried out in 18 h fasted male Swiss mice. Mice were allowed free access to pre-weighed food pellet (standard chow) after i.p.

injection of saline vehicle (0.9% (w/v) NaCl) or test peptides (25 or 250 nmol/kg bw). Cumulative food intake (g) was measured at 0, 30, 60, 90, 120, 150 and 180 minutes after peptide administration.

2.14.2 Acute effects of peptides on glucose tolerance

Glucose and insulin concentrations were measured in 18 h fasted male Swiss mice before and after i.p. injection of glucose alone (18 mmol/kg bw) or in combination with test peptides (25 nmol/kg bw), as well as test peptides together with GIP (25 nmol/kg bw). Blood samples were collected at various time points (0, 15, 30, 60 and 105 minutes) and glucose and insulin levels measured as stated in Section 2.15.

2.14.3 Persistent effects of peptides on glucose tolerance

In order to assess the duration of peptide action, delayed glucose tolerance tests were performed on 4 h fasted Swiss mice. Mice were i.p. injected with saline vehicle or test peptides (25 nmol/kg bw) at 2, 4, 8 or 12 h prior to administration of a i.p. glucose challenge (18 mmol/kg bw). Glucose was monitored at various time points (0, 15, 30, 60 and 105 minutes) as described in Section 2.15.

2.15 BIOCHEMICAL ANALYSIS

Blood samples were collected from the cut tip of the tail vein of conscious mice into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany). Blood samples were immediately centrifuged using Beckman centrifuge at 13,000 rpm for 3 minutes. Plasma was carefully aliquoted into fresh Eppendorf tubes and stored at -20 ^{o}C for further biochemical analysis. Blood glucose was measured directly using a Contour blood glucose meter (Bayer, Newbury, UK). Plasma and

pancreatic insulin were assayed using RIA [Flatt and Bailey, 1982]. For measurement of plasma insulin, 20 µl of test sample was diluted with 180 µl of working RIA buffer (1:10 dilution) and measured in duplicates by RIA, as outlined previously in Section 2.11.2. For measurement of pancreatic insulin content, 5 µl of test sample was diluted with 195 µl of working RIA buffer (1:40 dilution).

2.16 LONG TERM ANIMAL STUDIES

Two different models were used in sub-chronic assessment of peptides – HFF mice and HFF/STZ mice. Mice were grouped and maintained as detailed in Section 2.13.

2.16.1 Monitoring the effects of long term peptide administration

In chapter 3, groups of HFF mice (n=12) received twice daily (09:00 and 17:00 h) i.p. injections of saline vehicle or test peptides alone or in combination (each at 25 nmol/kg) for duration of test period. Additionally, in Chapter 3, following a 28-day treatment period, a sub-group (n=6) of HFF mice received further twice daily injections of saline alone for 14 days to determine effect of treatment withdrawal, with assessment of the same parameters as documented above.

In chapter 5, respective treatments were commenced on day 0 as follows in HFF mice; HFF mice (n = 8) received oral sitagliptin phosphate monohydrate once daily (50 mg/kg bw), intraperitoneal (i.p.) Ψ-xenin-6 twice daily (25 nmol/kg bw) or a combination of both compounds using the same dosing regimen for 18 days. All twice daily treatments were administered at 09:30 and 17:00 h, with once daily treatments delivered at 09:30 h. Saline vehicle (i.p. or oral) was administered as control at each time point either orally or intraperitoneally, as appropriate. Doses of sitagliptin and Ψ-

xenin-6 were chosen based on previous published studies in mice [Gault *et al.* 2015b*;* Craig *et al.* 2019]. We also employed a gold standard lean control group (n = 8) on normal chow diet without STZ injections. These control mice were also administered saline vehicle twice daily (orally at 09:30 and i.p at 17:00 h) throughout the study.

In chapter 6, on day 0, HFF mice (n = 10) received oral sitagliptin once daily (25 mg/kg bw), intraperitoneal (i.p.) TNP-470 once daily (1 mg/kg bw) or a combination of both compounds using the same dosing regimen for 18 days. All once daily treatments were administered at 11:00 h. Saline vehicle (i.p. or oral) was administered as control at 11:00 h either po or ip. Sub-optimal therapeutic doses of sitagliptin and TNP-470 were chosen [Lijnen *et al*. 2010; Joharapurkar *et al*. 2014], to facilitate extrapolation of more obvious benefits of combined treatment regimen.

Before the start of the treatment regimens above, all animals were given twice daily injections / oral gavage, where appropriate, of saline alone at 09:00 and 17:00 h for 5 days to acclimatise mice to handling and injection stress. Cumulative energy intake, water intake, body weight, non-fasting blood glucose and plasma insulin concentrations were monitored at regular intervals. Whole blood was taken for measurement of HbA1c concentrations as appropriate. Glucose and insulin concentrations were measured as described in Section 2.15.

2.16.2 Parameters assessed following long term studies

2.16.2.1 Glucose tolerance test

Fasted (18 h) mice received i.p. injections of glucose alone (18 mmol/kg bw). Blood samples were collected at various time points (0, 5, 15, 30 and 60 minutes (Chapter 5

& 6) or 0, 15, 30, 60, 105 and 120 minutes (Chapter 3)). Glucose and insulin concentrations were measured as described in Section 2.15.

2.16.2.2 Metabolic responses to acute administration of treatment regimens

In chapter 3, fasted (18 h) mice received i.p. injections of glucose (18 mmol/kg bw) in combination with respective treatment regimens (25 nmol/kg bw). Blood samples were collected at various time points (0, 15, 30, 60, 105 and 120 minutes). Glucose and insulin concentrations were measured as described in Section 2.15.

2.16.2.3 Pyruvate Tolerance Test

In chapters 5 and 6, fasted (18 h) mice received i.p. injections of sodium pyruvate (2 g/kg bw). Blood glucose was measured prior to (t=0) and 5, 15, 30 and 60 minutes after i.p. administration.

2.16.2.4 Insulin sensitivity test

Insulin sensitivity was performed in non-fasted mice. Blood glucose was measured prior to (t=0) and then 30 and 60 minutes after i.p. administration of bovine insulin (25 U/kg bw). To evaluate insulin resistance, HOMA-IR was calculated [Matthews *et al.* 1985]. HOMA-IR = fasting plasma glucose X fasting plasma insulin / 22.5. Fasting glucose and insulin were measured in mmol/l and mU/l, respectively.

2.16.3 Bone mineral density and body composition determination by dual energy X-ray absorption (DEXA)

In chapter 3, lean mass and total fat percentage was measured using the Piximus densitometer (Inside Outside Sales, Wisconsin, USA). Prior to measurements, the

instrument was calibrated using a manufacturer supplied protocol. Once quality control was passed, mice were culled by cervical dislocation and positioned on specimen tray following manufacturer's instructions.

2.16.4 Measurement of indirect calorimetry, energy expenditure and locomotor activity

The Comprehensive Laboratory Animal Monitoring System (CLAMS) with Oxymax system (Columbus Instruments, OH, USA) was used to assess standard parameters of energy expenditure, indirect calorimetry, locomotor activity and feeding patterns as described previously by Gault *et al*. (2011b). At the end of treatment period, mice (n=5) were placed into airtight metabolic chambers for 24 h. Mice were allowed to acclimatise to the new environment for 3 h prior to 21 h monitoring time period. Carbon dioxide and oxygen metabolism was monitored for 30 seconds at 15 min intervals for a total of 21 h. Respiratory exchange ratio was calculated by dividing volume of CO_2 produced by volume of O_2 consumed. Energy expenditure (EE) was calculated using RER with the following equation:

$$EE\ (Kcal/h) = (3.815 + 1.232 \times RER) \times VO_2$$

Feeding patterns were recorded using spring loaded feeding hoppers attached to a weigh balance. Locomotor activity was recorded using dual infrared (IR) beams-primary (X-axis) and secondary (Z-axis) IR beam. Animal motion leading to interruption of either infrared beam was represented as 'one count'. Counts in each axis were measured every minute for each mouse over 24 h time period. General movement of mice was measured in terms of X-beam and expressed in two ways: total

or ambulatory X-beam counts. Exploration activity of mice was expressed in terms of total Z-beam counts.

2.16.5 HbA1c measurement

Prior to and at the end of respective chronic study in chapter 3, HbA1c values were measured in whole blood using A1cNow^{+} kits (PTS diagnostics, IN, USA) as per standard manufacturer's instructions.

2.16.6 Measurement of pancreatic insulin content

Following chronic studies, pancreatic tissue was excised and divided longitudinally. Half of the tissue was kept for histological analysis (Section 2.16.7) and half immediately snap frozen for subsequent protein extraction. Pancreata were weighed and homogenized, using a VWR VDI 12 handheld homogenizer (VWR, UK), in ice-cold acid ethanol (1.5% HCl, 75% ethanol and 23.5% distilled H_2O). Samples were centrifuged at 3,000 rpm for 20 minutes at 4 ^{o}C. Supernatant was transferred to a 15 ml centrifuge tube, 10 ml TRIS buffer (pH 7.4) added and then concentrated to dryness using AES1000 Speed-Vac concentrator. Samples were re-suspended in 200 µl of acetonitrile and 1.8 ml TRIS buffer and stored at -20 ^{o}C until further analysis. For measurement of insulin content of extracts, 20 µl of test sample was diluted with 180 µl of working RIA buffer (1:10 dilution) and measured using RIA (Section 2.11.2). Total protein content was measured by Bradford assay. Briefly, tissue extract (5 µl; in duplicate) was aliquoted into a 96-well plate and 250 µl of Bradford reagent (Sigma Aldrich, Poole, Dorset, UK) added. The plate was incubated at room temperature for 15 minutes. Absorbance was read at 595 nm using a microplate reader (Molecular Devices, Sunnyvale, USA). Amount of protein in extract was estimated using the

linear range of the generated reference curve of BSA standards (concentration ranging from 0. – 2 mg/ml).

2.16.7 Immunohistochemistry

Pancreatic tissue was excised, divided longitudinally and processed for immunohistochemical examination. Pancreatic tissues were fixed in 4% paraformaldehyde at 4 ºC for 48 h, to preserve cellular architecture. Pancreata were then embedded in paraffin wax and processed using an automated tissue processor (Leica TP1020, Leica Microsystems, Nussloch, Germany). Concisely, this involved dehydrating tissues in increasing concentrations of ethanol (70 – 100%), before immersion in xylene to remove alcohol and allow for embedding in paraffin wax. Once processed, tissues were cut at 7 μm thickness using a microtome (Shandon finesse 325, Thermo scientific, UK) and sections selected at an interval of 10 sections. Immunoreactive staining for insulin and glucagon was assessed by firstly, deparaffinising tissue sections by immersing in xylene (20 minutes; Sigma Aldrich, Dorset, UK). Sections were then rehydrated through a series of decreasing ethanol concentrations (100%, 95%, 85%, 70% and 50%). After antigen retrieval at 94 ºC for 20 minutes using sodium citrate buffer (10 mmol/l Sodium Citrate, 0.05% Tween 20, pH 6.0), slides were allowed to cool (20 minutes) before being blocked using 3% BSA for 1 h at room temperature. Sections were then incubated overnight at 4 ºC with primary antibodies: mouse anti-insulin antibody (1:500; Abcam, ab6995) and guinea-pig anti-glucagon antibody (PCA2/4, 1:400; raised in-house). Following overnight incubation, excess antibody was removed by washing with PBS three times. Slides were then incubated with appropriate secondary antibody; Alexa Fluor 488 goat anti-guinea pig IgG (1:400) or Alexa Fluor 594 goat anti-mouse IgG (1:400), for 45

minutes at 37 ^{o}C. Slides were again rinsed with PBS and stained with nuclear stain-DAPI (4, 6-diamidino-2-phenylindole; 300 nmol/l in PSB) for 15 minutes at 37 ^{o}C. Finally, slides were washed with PBS before mounting slides using aqueous mounting medium (Glycerol: PBS – 1:1) and coverslips. Stained slides were viewed under a FITC (488 nm), TRITC filter (594 nm) or DAPI filter using a fluorescent microscope (Olympus system microscope, model BX51) and photographed using a linked DP70 camera adapter system on Cell^{F} software. Islet parameters were analysed using Cell^{F} image analysis software (Olympus Soft Imaging Solutions, GmbH) as outlined in section 2.17.

For Ki67 staining, tissue sections were deparaffinised, rehydrated and probed with primary antibodies: mouse anti-insulin antibody (1:400; Abcam, ab6995), guinea-pig anti-glucagon pig anti-glucagon antibody (PCA2/4, 1:400; raised in-house), or rabbit anti-Ki67 (1:400; Abcam ab16667), as appropriate. Sections were then incubated with secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (1:200), Alexa Fluor 488 goat anti-guinea-pig IgG (1:200; Abcam ab150185) or Alexa Fluor 594 goat anti-rabbit IgG (1:200) respectively. The slides were viewed under a FITC (488 nm) or TRITC filter (594 nm) and analysed as detailed above.

2.16.8 Measurement of plasma and pancreatic GIP and GLP-1 content

Total GLP-1 and GIP content in plasma were determined using specific ELISA following manufacturer's instructions (GLP-1 Total ELISA, EZGLP-1T-36K, Millipore; rat/mouse GIP (Total) ELISA, EZRMGIP-55K, Millipore). The amount of total GIP in each sample was estimated using a reference curve of known GIP

standards (8.2 pg/ml–2000 pg/ml). The amount of total GLP-1 was estimated using a reference curve of known GLP-1 standards (4.9 pM–1200 pM).

2.16.9 Measurement of plasma and intestinal xenin content

Xenin content in plasma and intestinal tissue extracts was determined using xenin chemiluminescent assay (FEK-046-74, Phoenix Pharmaceuticals), following manufacturer's instructions. The amount of xenin in the sample was estimated using a reference curve of known xenin standards (1 pg/ml–1,000,000 pg/ml).

2.1.10 DPP-4 Activity

DPP-4 activity of terminal plasma, as well as sitagliptin and Ψ-xenin-6 alone and in combination *in vitro* (both at 10^{-6} mol/l), was determined using a fluorometric assay that measures free 7-amino-4-methyl-coumarin (AMC) liberated from Gly-Pro-AMC substrate [Lindsay *et al*. 2005; Harnedy and Fitzgerald, 2013]. Briefly, 10 µl of plasma (n=8) or peptide (n=4) sample, where appropriate, was incubated with 30 µl 0.02 mol/l Tris– HCl buffer, pH8.0 containing 0.10 mol/l NaCl and 1 mmol/l EDTA, and 50 µl, 200 µM H-Gly-Pro-AMC. Following 5 min incubation at 37 °C the reaction with the peptide samples were initiated by addition of 10 µl DPP-4 (8 mU mL^{-1}). The change in fluorescence was monitored over a 30 min period using a plate reader at excitation and emission wavelengths of 360 and 460 nm, respectively.

2.16.11 mRNA extraction and conversion to cDNA

mRNA was extracted from excised liver tissue. Briefly, liver tissues were homogenised in 1 ml TRIZOL reagent per 100 mg of tissue using a power homogenizer, with 200 µl of chloroform per 1 ml of TRIZOL reagent added. Samples

were vortexed vigorously for 15 seconds and incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4ºC. Following centrifugation, the mixture separated into a lower red, phenolchloroform phase, an interphase, and a colourless upper aqueous phase, with RNA remaining exclusively in the aqueous phase. This upper aqueous layer was transferred into a fresh tube, with 500 µl of isopropanol per 1 ml of TRIZOL reagent added. Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 20 minutes at 4ºC, with the RNA precipitate forming a gel-like pellet at the bottom of the tube. Supernatant was removed and the pellet washed with 75% ethanol (1 ml of ethanol per 1 ml of TRIZOL reagent used) and centrifuged at 12,000 x g for 5 minutes at 4ºC. The above wash was repeated three times, with the RNA pellet allowed to air dry for 10 minutes. Pellet was re-suspended in 20 µl nuclease free water, and incubated at 60-70ºC for 5 minutes and then placed on ice. Concentration and purity of extracted mRNA was determined using nanophotometer. Each RNA sample (3 µg) was converted to cDNA at 42°C for 50 min in the presence of 0.5 µg oligo dT(12–18) primer, 10 mmol/l dNTP and 200 U Superscript II reverse transcriptase (Invitrogen, Paisley, UK) in a final volume of 20 µl using a GeneStorm GS1 Thermal Cycler (Gene Technologies Ltd, Essex, UK). Concentration of cDNA was determined using the nanophotometer and cDNA was stored at -20ºC.

2.16.12 Real-time reverse transcription polymerase chain reaction (RT-PCR)

The reaction mix consisted of 4.5 µl of SYBR green, 1 µl of each of the primers (forward and reverse), 3 µl RNA free water and 1 µl of respective cDNA. β-actin (*ACTB*) was used as an internal control for normalisation. Reactions took place in Lightcycler 96 multi-well plates. PCR conditions were 95 °C for 10 min, followed by

cDNA amplification for 45 cycles with 95 °C denaturation for 10 s, 60 °C annealing for 30 s and 72 °C elongation for 1 s, followed by 30 s cooling at 40 °C. Relative quantification using the $2^{-\Delta\Delta CT}$ (two delta, delta comparative test) method was used to calculate differences in gene expression between samples. mRNA levels of all genes were normalised to positive control for PCR (β-actin – reference gene). (See table 2.2)

2.17 IMAGE ANALYSIS

Images were analysed using Cell^F image analysis software (Olympus Soft Imaging Solutions, GmbH) to assess: islet area, beta cell area and alpha cell area (expressed as μm^2). For islet size distribution, islets were defined as small, medium or large (<10,000 μm^2, 10,000-25,000 μm^2 or >25,000 μm^2 respectively). Proliferation frequency analysis in beta cells was quantified using Ki-67 co-stained with insulin. Beta cell apoptosis was quantified by counting the number of TUNEL positive cells and expressed as a percentage of cells analysed.

2.18 STATISTICAL ANALYSIS

GraphPad PRISM (Version 5) was used to carry out statistical analysis. Groups of data were considered to be significantly different if $P<0.05$. Results are expressed as mean ± SEM and data compared using one-way repeated measures ANOVA followed by the Student-Newman-Keuls *post-hoc* test. The unpaired student *t*-test was used where appropriate. Area under curve (AUC) and area above curve (AAC) analyses were calculated using appropriate baseline subtraction.

Table 2.1 Structure of peptides used in Chapter 3, 4 and 5

Name	Purity	Sequence
(DAla^2)GIP/Xenin-8-Gln	>95%	Y[DA]EGTFISDYSIAMHPQQPWIL-OH
Xenin-6	>95%	H-KRPWIL-OH
Xenin-6-NH_2	>95%	H- KRPWIL-NH_2
Xenin-6-Gln	>95%	H-QQPWIL-OH
Xenin-6-Gln-NH_2	>95%	H-QQPWIL-NH_2
Ψ-Xenin-6	>95%	H-K-(CH_2NH)-RPWIL-OH

Table 2.2: Primers used for qPCR.

Mouse: Gene nomenclature	Primer Sequence
PEPCK	Forward 5' – 3' TTGAACTGACAGACTCGCCC Reverse 5' – 3' GGCACTTGATGAACTCCCCA
G6Pase	Forward 5' – 3' GGACCTCCTGTGGACTTTGG Reverse 5' – 3' TGCTGAGTTCTCCCTTGCAG
IRS-1	Forward 5' – 3' AGGACCTCACGTCTTCCTCTT Reverse 5' – 3' TTCCGGTGTCACAGTGCTTTC
FGF21	Forward 5' – 3' AGATGGGCTCTCTATGGATCG Reverse 5' – 3' GGGCTTCAGACTGGTACACAT
AKT1	Forward 5' – 3' GCCGCCTGATCAAGTTCTCC Reverse 5' – 3' CAGCGCATCCGAGAAACAAAAC
β-actin	Forward 5' – 3' CCTCACTGTCCACCTTCCAG Reverse 5' – 3' AGGGTGTAAAACGCAGCTCA

Chapter 3

Persistent antidiabetic benefits of GIP/xenin hybrid in combination with exendin-4 on glucose tolerance and islet architecture in HFF mice

3.1 SUMMARY

Antidiabetic therapeutics that focus on targeting multiple signalling pathways have gained more interest in the treatment of T2DM. In this present chapter, we created a treatment paradigm of three key gastrointestinal hormones, integrating the antidiabetic benefits of GIP, xenin and GLP-1 signalling pathways. The current study has determined the ability of exendin-4 to augment the antidiabetic benefits of the recently characterised GIP/xenin hybrid, (DAla^2)GIP/xenin-8-Gln, in high fat fed (HFF) mice, as well as examining persistence of beneficial metabolic effects in HFF mice. Twice-daily injection of (DAla^2)GIP/xenin-8-Gln for 28 days in HFF mice significantly reduced energy intake ($P<0.05$), body weight ($P<0.001$), circulating glucose ($P<0.05$) and HbA1c ($P<0.05$ - $P<0.01$) levels. Glucose tolerance and insulin sensitivity was also significantly ($P<0.05$ - $P<0.01$) improved by day 28. Overall pancreatic islet, beta- and alpha-cell areas were reduced ($P<0.001$), with concurrent reduction ($P<0.001$) in alpha- and beta-cell proliferation that was more apparent in combined treatment group ($P<0.05$). Addition of exendin-4 to (DAla^2)GIP/xenin-8-Gln therapy did not significantly improve metabolic control. Remarkably, beneficial effects were still evident 14 days following complete cessation of peptide administration. Thus, circulating glucose and insulin, HbA1c concentrations and glucose tolerance were still significantly improved when compared to control HFF mice on day 42, with minimal changes to islet architecture. These studies highlight the clear and persistent metabolic advantages of sustained activation of GLP-1 receptors, together with simultaneous activation of related GIP and xenin signalling pathways in diabetes.

3.2 INTRODUCTION

The focus of new therapeutic interventions for T2DM is moving towards a combinational targeting approach, in a bid to develop drugs capable of simultaneously correcting the multiple dysregulated metabolic pathways associated with the disorder [Irwin *et al*. 2015; Mansur *et al*. 2016; Tschöp *et al*. 2017; Hasib *et al*. 2018a, 2018b]. One of the major instigating factors behind this initiative correlates with knowledge that, in many patients, the rapid remission of T2DM following certain types of bariatric surgeries is linked to changes in the secretion and action of numerous intestinal-derived peptide hormones [Cummings. 2009; Dimitriadis *et al*. 2017; Meek *et al*. 2016; Su *et al*. 2016]. Thus, combined administration of therapeutically viable gut peptide entities improves metabolic control beyond that observed with a single peptide hormone [Bhat *et al*. 2013]. Hybrid gut peptides, designed as single molecules capable of modulating multiple hormone receptors, have shown particular promise [Gault *et al*. 2013; Bhat *et al*. 2013; Irwin *et al*. 2015; Tschöp *et al*. 2016; Hasib *et al*. 2018a, 2018b]. Indeed, a dual GIP and GLP-1 receptor agonist hybrid has been shown to display good initial efficacy for T2DM in early clinical trials [Frias *et al*. 2017]. This observation indicates that the impaired insulinotropic activity of GIP reported in humans with T2DM [Nauck *et al*. 1993] can be reversed. It follows that approaches to enhance GIP action could lead to even greater beneficial therapeutic effects for T2DM.

In this regard, xenin, a peptide hormone co-secreted from the same enteroendocrine K-cell as GIP, has been shown to potentiate the insulin releasing actions of GIP [Wice *et al*. 2010; Taylor *et al*. 2010; Wice *et al*. 2012; Martin *et al*. 2014; Gault *et al*. 2015; Parthsarathy *et al*. 2016]. Additional studies have also highlighted independent beneficial effects of xenin on satiety as well as pancreatic beta cell function and

survival [Leckstrom *et al*. 2009; Gault *et al*. 2015; Craig *et al*. 2018]. Recent studies have additionally characterised dual- and triple-acting hybrid peptides, with a GLP-1 backbone, that also contain a xenin component [Hasib *et al*. 2017; 2018a; 2018b]. However, since xenin is known to potentiate the action of GIP [Craig *et al*. 2018], combining the bioactive regions of GIP and xenin into a single fusion hybrid peptide would seem more appealing. Indeed, our laboratory has already documented the antidiabetic benefits of a GIP/xenin hybrid peptide, namely (DAla^2)GIP/xenin-8-Gln [Hasib *et al*. 2017], that integrates enzymatically stable forms of the N-terminally biologically active domain of GIP [Hinke *et al*. 2001] with the C-terminal bioactive region of xenin [Silvestre *et al*. 2003; Martin *et al*. 2014]. (DAla^2)GIP/xenin-8-Gln was shown to restore GIP sensitivity, enhance beta cell function and improve glucose homeostasis following sustained treatment in HFF mice [Hasib *et al*. 2017]. Although GIP/xenin induced notable positive effects in HFF mice, many metabolic parameters were still abnormal in these rodents, suggesting potential for further improvements.

In the present study we have employed combined treatment of (DAla^2)GIP/xenin-8-Gln, with the clinically approved long-acting GLP-1 mimetic exendin-4, to create a treatment paradigm that integrates the established antidiabetic benefits of GIP, xenin and GLP-1 signalling [Irwin and Flatt 2015; Craig *et al*. 2018]. Exendin-4 was chosen based on knowledge of the positive biological interactions between GLP-1 with both GIP and xenin [Irwin *et al*. 2009; Hasib *et al*. 2018a]. Given the structure of (DAla^2)GIP/xenin-8-Gln, and the site of the main bioactive regions within GIP, xenin and GLP-1 [Hinke *et al*. 2001; Martin *et al*. 2016], it was not possible to create a triple-acting GIP/xenin/GLP-1 hybrid molecule. As such, antidiabetic effects of 28 days twice-daily treatment of (DAla^2)GIP/xenin-8-Gln in combination with exendin-

4 were examined in an environmentally induced mouse model of type 2 diabetes, namely HFF mice. This model mimics many of the major pathophysiologies of human type 2 diabetes including obesity, hyperinsulinemia, and pancreatic beta cell demise [King and Austin, 2017]. In addition, to assess the potential for more persistent beneficial effects of the treatment paradigms, the consequence of cessation of all treatments for a further 14 days was also studied. The results emphasise that prolonged modulation of GIP, xenin and GLP-1 signalling pathways represents a novel and exciting treatment option for type 2 diabetes, with enduring positive metabolic effects.

3.3 MATERIALS AND METHODS

3.3.1 Peptides

All peptides were obtained as described (Section 2.2). Peptides were confirmed pure by HPLC (Section 2.3.1) and characterised using MALDI-TOF MS (Section 2.3.2).

3.3.2 Animals

Sub chronic studies were performed using male Swiss mice (10-12 weeks). All animals were maintained as described previously in Sections 2.13 and 2.13.2.

3.3.3 Sub chronic *in vivo* studies

Over a 28-day period, HFF mice received twice daily (09:30 and 17:00 h) injections of saline vehicle, (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw; i.p.), exendin-4 (25 nmol/kg bw; i.p.), or a combination of both peptides, as described in section 2.16.1. Energy intake, body weight, blood glucose and plasma insulin concentrations were assessed at regular intervals. Whole blood was taken for measurement HbA1c as

appropriate (Section 2.16.5). At the end of the treatment period glucose tolerance and insulin sensitivity tests were performed. In addition, metabolic response to acute administration of respective treatment regimens was examined (see Sections 2.16.2.1, 2.16.2.2 and 2.16.2.4). Terminal analyses included measurement of total fat mass by DEXA scanning (Section 2.16.3) and extraction of pancreatic tissue with appropriate processing for measurement of hormone content following acid/ethanol extraction or islet architecture (Section 2.16.6). For proliferation studies, insulin or glucagon stained slides were co-incubated at 37 °C with rabbit anti-Ki-67 primary antibody (Section 2.16.7). Finally, on day 28 observations were continued in a sub-group (n=6) of mice following cessation of treatment regimens for a further 14 days, with assessment of the same parameters as documented above.

3.3.4 Biochemical analysis

Blood samples were collected (Section 2.15), and glucose and insulin assayed (Sections 2.11.2 and 2.15). HbA1c was measured as described in Section 2.16.5.

3.3.5 Statistical analysis

Statistical analysis was completed using GraphPad PRISM (Section 2.18).

3.4 RESULTS

3.4.1 Effect of 28-day peptide administration, as well as 14-day cessation of treatment, on energy intake, body weight, HbA1c, circulating glucose and insulin in HFF mice.

On day 28, body weight change was significantly ($P<0.001$) reduced in all HFF treatment groups when compared to saline controls (Figure 3.1A). However,

following 14-days cessation of treatment, effects on body weight were reversed (Figure 3.1A). There was no change in body fat masses during or after the treatment interventions (Figure 3.1B). Interestingly, (DAla^2)GIP/xenin-8-Gln and exendin-4, but not combined treatment, significantly ($P<0.05$) reduced cumulative energy intake over the 28 day period, with this effect fading by day 42 (Figure 3.1C). HbA1c was reduced ($P<0.05$ - $P<0.01$) in all treatment groups on days 28 and 42 (Figure 3.1D). In keeping with this, non-fasting glucose levels were also significantly ($P<0.05$) lower in (DAla^2)GIP/xenin-8-Gln and exendin-4 treatment groups on day 15 (Figure 3.2A), and in all HFF treatment groups ($P<0.05$ - $P<0.01$) on days 28 and 42 (Figure 3.2A). Corresponding insulin levels were decreased ($P<0.05$) in (DAla^2)GIP/xenin-8-Gln and exendin-4 treated mice on day 28, and in all HFF treatment groups ($P<0.001$) on day 42 (Figure 3.2B).

3.4.2 Effect of 28-day peptide administration, as well as 14 day cessation of treatment, on glucose tolerance, acute metabolic response to respective peptide treatment, insulin sensitivity and pancreatic insulin content in HFF mice.

Twice-daily treatment with exendin-4 in HFF mice resulted in a reduction ($P<0.05$) in glucose levels at 105 min following an i.p. glucose load on day 28 (Figure 3.3A). The other treatment interventions, namely (DAla^2)GIP/xenin-8-Gln alone and in combination with exendin-4, had a tendency to decrease time-dependent glucose levels following glucose challenge, but this failed to reach significance (Figure 3.3A). However, overall 0-120 min glucose AUC values were significantly ($P<0.05$ - $P<0.01$) decreased in all treatment groups when compared to saline controls (Figure 3.3A). Similarly, corresponding AUC insulin secretory responses were reduced ($P<0.05$ - $P<0.01$) in all HFF treatment groups (Figure 3.3B). An identical glucose

challenge on day 42, following 14-days treatment cessation, was associated with decreased ($P<0.05$) individual and AUC glucose values in treatment groups when compared to saline controls (Figure 3.3C), with no obvious changes in glucose-induced insulin secretion (Figure 3.3D). In terms of the acute metabolic effects of the same peptide treatment in each respective group on day 28, (DAla^2)GIP/xenin-8-Gln had no impact on glucose disposal or insulin secretion when compared to saline controls (Figure 3.4A,B). In contrast, exendin-4 alone, and in combination with (DAla^2)GIP/xenin-8-Gln, retained impressive ($P<0.001$) glucose-lowering effects in their respective treatment groups (Figure 3.4A). The beneficial glucose homeostatic actions of combination treatment were associated with reduced ($P<0.01$) insulin levels (Figure 3.4B). Strikingly similar acute metabolic effects of each of the peptide treatments were noted on day 42, following 14-days treatment cessation (Figure 3.4C,D).

Further to this, following administration of exogenous insulin on days 28 and 42, individual glucose levels were non-significantly decreased in all HFF treatment groups when compared to saline controls (Figure 3.5A,B). Furthermore, 28 days treatment with exendin-4 alone and in combination with (DAla^2)GIP/xenin-8-Gln significantly ($P<0.01$) augmented the overall hypoglycaemic action over the 60 min observation period (Figure 3.5A). A similar effect ($P<0.05$) was also noted on day 42 in mice previously treated with exendin-4 for 28 days mice (Figure 3.5B). Analysis of pancreatic insulin on days 28 and 42 revealed elevations ($P<0.05$ - $P<0.001$) in all treatment groups when compared to saline controls (Figure 3.5C).

3.4.3 Effect of 28-day peptide administration, as well as 14-day cessation of treatment, on pancreatic islet histology in HFF mice.

Visual inspection of immunofluorescently stained pancreatic islets on day 28 revealed no obvious abnormalities in saline or peptide treated HFF mice (Figure 3.6A-D). Interestingly, twice daily treatment with (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides significantly ($P<0.01$ - $P<0.001$) decreased overall islet and alpha cell areas when compared to saline control HFF mice (Figure 3.6E,F). Beta cell area was also reduced ($P<0.001$ and $P<0.05$, respectively) in HFF mice treated with exendin-4 alone, or in combination with (DAla^2)GIP/xenin-8-Gln, but was increased in (DAla^2)GIP/xenin-8-Gln treated mice when compared to exendin-4 alone treated mice (Figure 3.6G). These islet architectural changes were related to reductions ($P<0.001$) in both alpha- and beta cell proliferation in all treatment groups of mice (Figure 3.6H,I), with decreased alpha cell proliferation most apparent in HFF mice treated with a combination of (DAla2)GIP/xenin-8-Gln and exendin-4. Beta to alpha cell ratio was significantly ($P<0.05$ - $P<0.001$) increased by all treatments when compared to HFF controls, but reduced ($P<0.05$ - $P<0.01$) in the combined treatment group when compared to (DAla^2)GIP/xenin-8-Gln alone (Figure 3.6J). Essentially similar observations as day 28 were noted following 14-days cessation of treatment, with some small exceptions (Figure 3.7A-D). As such, HFF mice treated with (DAla^2)GIP/xenin-8-Gln and exendin-4 no longer presented with increased beta to alpha cell area (Figure 3.7J).

3.5 DISCUSSION

In agreement with previous reports [Hasib *et al*. 2017], sustained administration of (DAla^2)GIP/xenin-8-Gln to HFF mice resulted in prominent benefits on several

metabolic parameters. This included substantially reduced hyperglycaemia and glycated haemoglobin concentrations, improved glucose tolerance and enhanced beta cell function. Assessment of metabolic rate in these mice would have also been useful to understand underlying mechanisms. For example, exendin-4 therapy alone appeared to be the most effective intervention in terms of reducing body weight, despite no obvious changes in energy intake between groups of HFF mice. However, similar, although less striking, antidiabetic effects were previously observed in *db/db* mice [Craig *et al*. 2019], indicating that the positive effects of (DAla^2)GIP/xenin-8-Gln are transferable across diverse aetiologies of T2DM [King and Austin, 2017]. This is important given the complex nature of T2DM in humans [Prasad and Groop, 2015], and consistent with the positive outcomes of combined activation of GIP and xenin cell signalling pathways in diabetes [Hasib *et al*. 2017].

In an attempt to augment these advantageous effects, combination therapy of (DAla^2)GIP/xenin-8-Gln with the clinically approved GLP-1 mimetic, exendin-4, was employed. In HFF mice, exendin-4 exhibited a slight tendency to enhance the efficacy of (DAla^2)GIP/xenin-8-Gln, but this failed to reach significance for any of the parameters examined. Although circulating glucose was consistently reduced by all treatment interventions, an overnight fast resulted in similar glycaemic status in all HFF mice. Thus, the good efficacy of (DAla^2)GIP/xenin-8-Gln alone [Hasib *et al*. 2017], the relatively mild form of T2DM presented in HFF mice [King and Austin, 2017] and the fact that (DAla^2)GIP/xenin-8-Gln and exendin-4 may function through similar or complementary mechanisms [Irwin and Flatt, 2015; Craig *et al*. 2018], may preclude additive effects. More interestingly however, the beneficial effects of (DAla^2)GIP/xenin-8-Gln and exendin-4 combination therapy in HFF mice were

consistently associated with decreased insulin levels. Thus, despite the normal insulin secretagogue action of GIP, xenin and exendin-4 [Feurle *et al*. 1992; Green *et al*. 2003; Irwin *et al*. 2006b], beta cell function was suppressed but normal glycaemia maintained, as a direct result of amelioration of peripheral insulin resistance, as observed following a bolus insulin injection. Consistent with this view, islet and beta cell areas were reduced in these mice, most likely due to decreased insulin demand. Interestingly, both alpha- and beta cell proliferation were concomitantly decreased by all treatment regimens in HFF mice, with similar observations noted following cessation of treatment. Combined therapy was associated with the greatest reductions in alpha cell proliferation on days 28 and 42, which could partly explain the changes in beta:alpha cell ratio noted in this group of mice. However, earlier observations of important insulin-independent glucose lowering actions of GIP, xenin and GLP-1 receptor activation are also likely to be significant in the improved metabolic status of all treatment groups of mice [Irwin and Flatt, 2015; Craig *et al*. 2018]. Additional studies to directly assess glucose uptake and disposal in metabolically active tissues such as adipocytes, hepatocytes and myocytes would be required to confirm this.

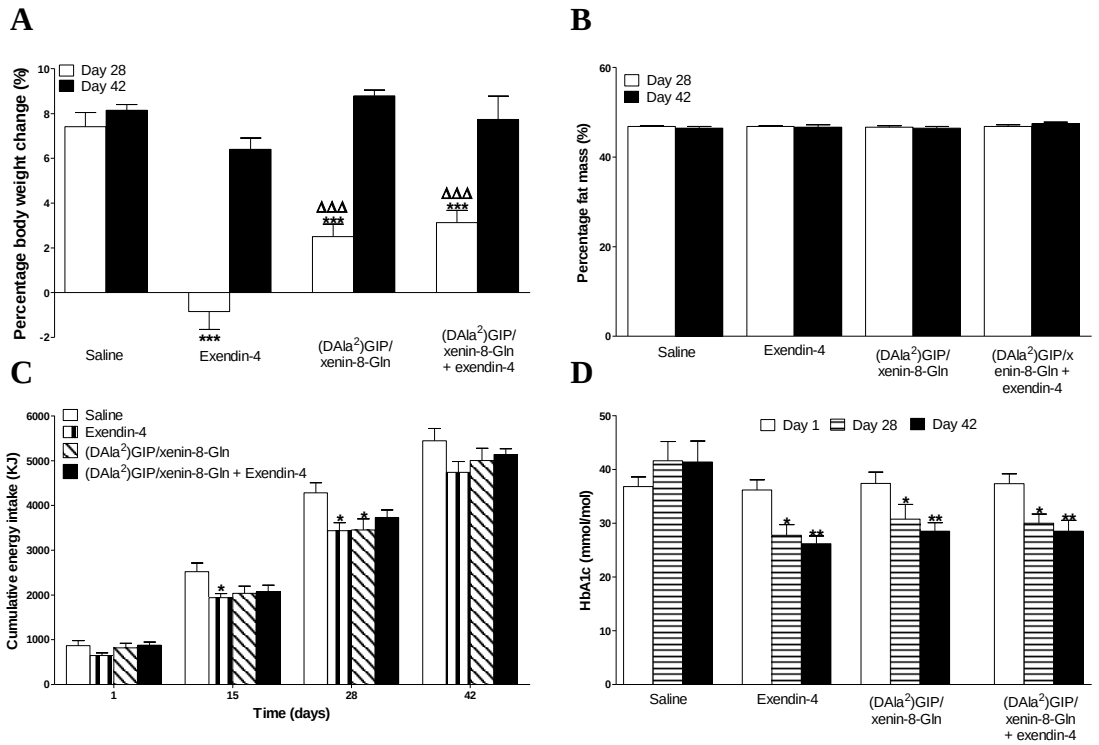
Nonetheless, on day 28 the advantageous glucose-lowering effects of acute injection of (DAla^2)GIP/xenin-8-Gln together with exendin-4 were confirmed in the combined treatment group, and associated with a substantially reduced insulin secretory response. This observation also rules out any potential issues around receptor desensitisation in the combination treatment, which has been suggested for GIP-based therapeutics in T2DM [Pathak *et al*. 2014]. However, assessment of GIP and GLP-1 receptor gene and protein expression in beta cells would also be interesting to assess in this regard. Nonetheless, the acute glucose-lowering and insulin secretory actions

of (DAla^2)GIP/xenin-8-Gln were somewhat less impressive than anticipated in HFF mice previously treated with this peptide for 28 days.

Given the progressive nature of T2DM [Fonseca, 2009], an extremely important observation from the current study was the endurance of beneficial metabolic effects following 14 days discontinuation of treatments in HFF mice. Thus, it is remarkable that circulating glucose concentrations and glucose tolerance remained substantially improved in all HFF treatment groups when compared to saline controls on day 42, and particularly so in the combination treatment group. This occurred despite no elevations in associated insulin concentrations and a progressive increase in body weight. It would imply favourable metabolic reprogramming in these mice, independent of energy balance that persists despite treatment withdrawal. Such positive effects have previously been shown with other gut hormone based therapies [Irwin *et al*. 2013a; Lynch *et al*. 2014]. Moreover, long lasting metabolic advantages are also a notable therapeutic advantage of the thiazolidinedione antidiabetic class of drugs [Gegick and Altheimer, 2004], and could represent a key benefit of combined (DAla^2)GIP/xenin-8-Gln and exendin-4 therapy. The lack of major changes in pancreatic islet architecture and insulin sensitivity following discontinuation of treatments is also indicative of preservation of improved metabolic state. Accordingly, the robust glucose homeostatic action of combined (DAla^2)GIP/xenin-8-Gln and exendin-4 therapy was associated with a significantly reduced insulin secretory response on day 42, similar to observations on day 28, highlighting sustainable benefits of this treatment approach for T2DM.

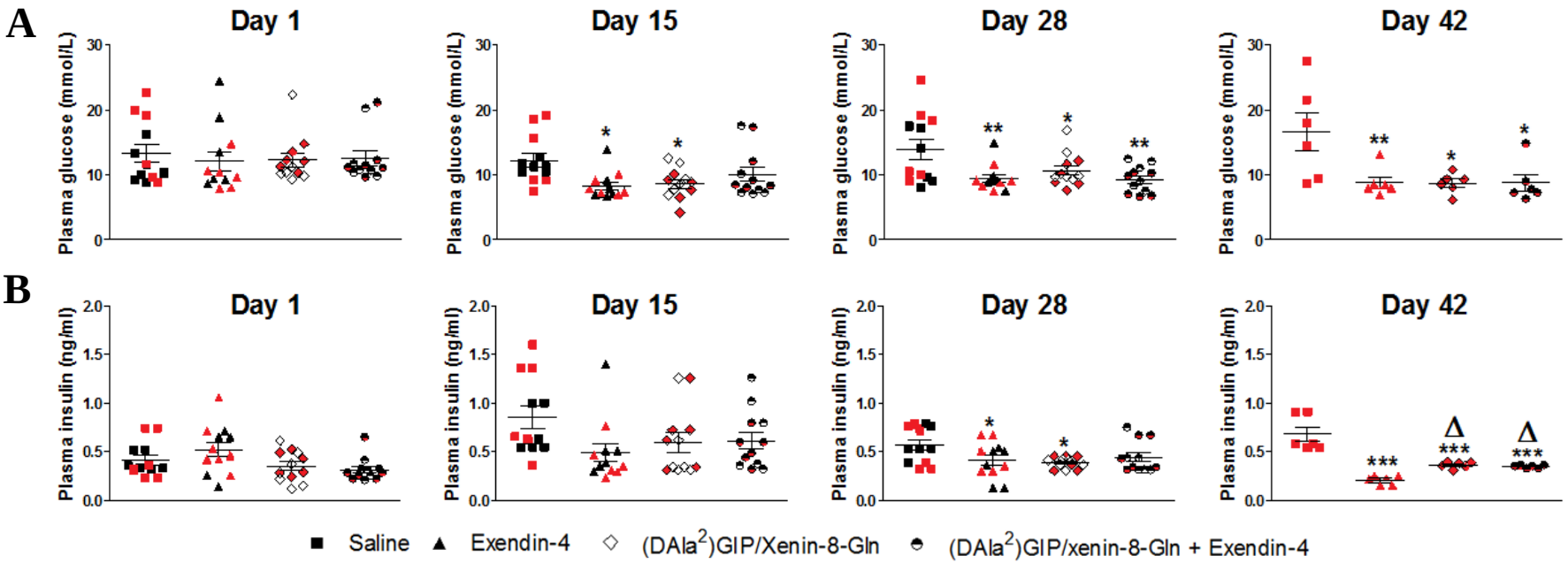
In conclusion, the present study confirms the antidiabetic attributes of (DAla^2)GIP/xenin-8-Gln in a HFF model of T2DM, with favourable effects augmented by combined therapy with exendin-4. Prominently, metabolic advantages of (DAla^2)GIP/xenin-8-Gln and exendin-4 therapy were remarkably persistent in nature in HFF mice, suggesting enduring positive antidiabetic effects. Taken together, it is clear that novel therapies including a GIP, xenin and GLP-1 component may have clinically exploitable benefits for human T2DM that merit further investigation. Accordingly, shorter xenin analogues may yet hold further therapeutic promise for T2DM, with smaller peptides potentially enabling non-injectable drug delivery and enhancing patient compliance moving towards the clinic.

Figure 3.1. Effects of (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides on (A) body weight change, (B) body fat mass (C) energy intake and (D) HbA1c in HFF mice.



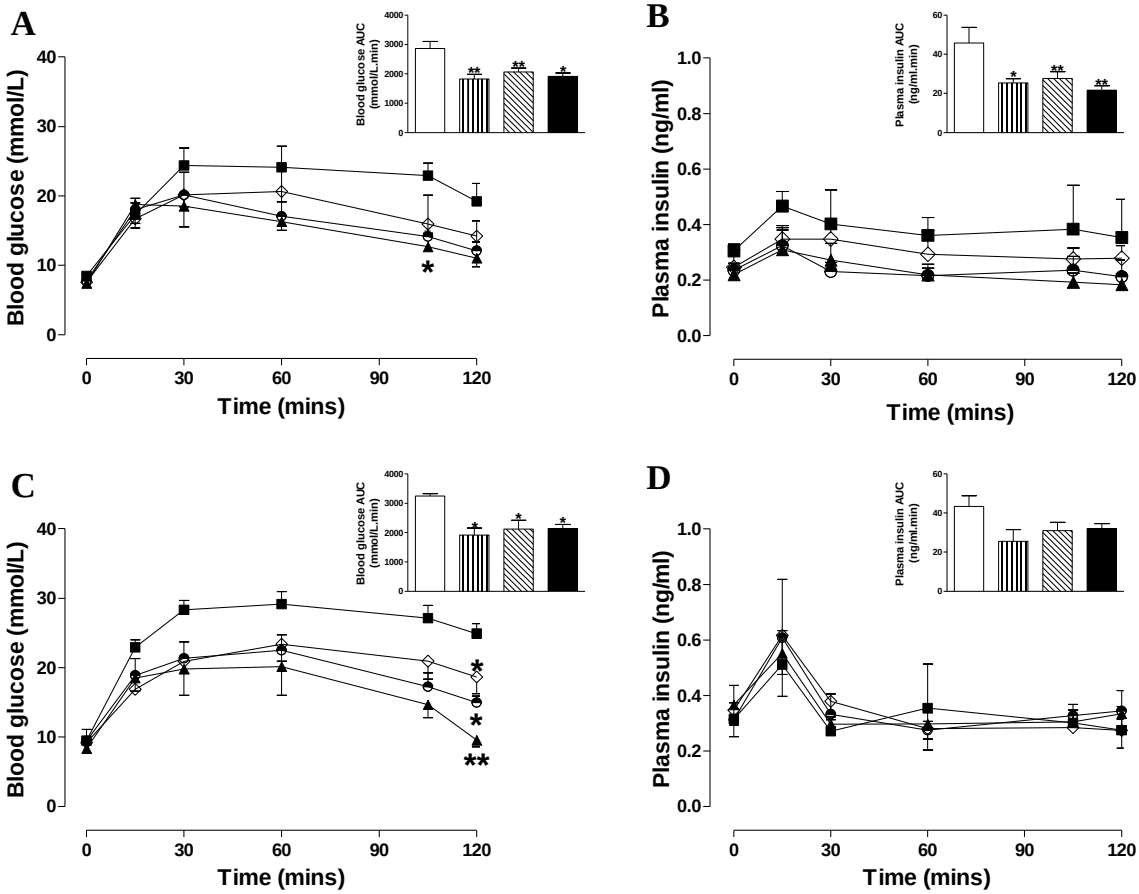
Parameters were measured at regular intervals 28 days during, and 14 days following cessation, of twice daily treatment with (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides, as appropriate. All values are mean ± SEM for twelve (28 days treatment) or six (14 days cessation of treatment) mice. *P<0.05, **P<0.01 and ***P<0.001 compared with HFF saline controls. $^{\triangle\triangle\triangle}$P<0.001 compared with exendin-4 alone.

Figure 3.2. Effects of (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides on (A) non fasting glucose and (B) insulin in HFF mice.



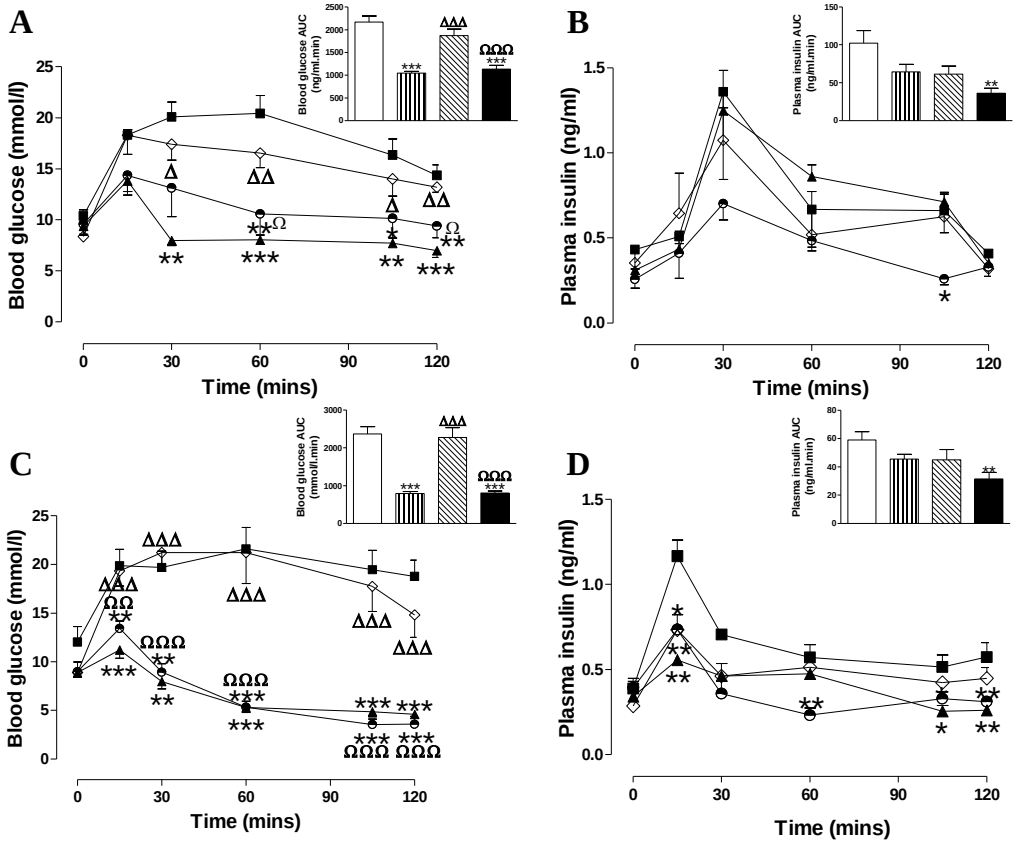
Parameters were measured at regular intervals 28 days during, and 14 days following cessation, of twice daily treatment with (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides, as appropriate. The n = 6 mice randomly selected to progress to cessation of treatment for 14 days are depicted by the red symbols. All values are mean ± SEM for twelve (28 days treatment) or six (14 days cessation of treatment) mice. *P<0.05, **P<0.01 and ***P<0.001 compared with HFF saline controls. $^{\Delta}$P<0.05 compared with exendin-4 alone.

Figure 3.3. Effects of (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides on glucose tolerance in HFF mice.



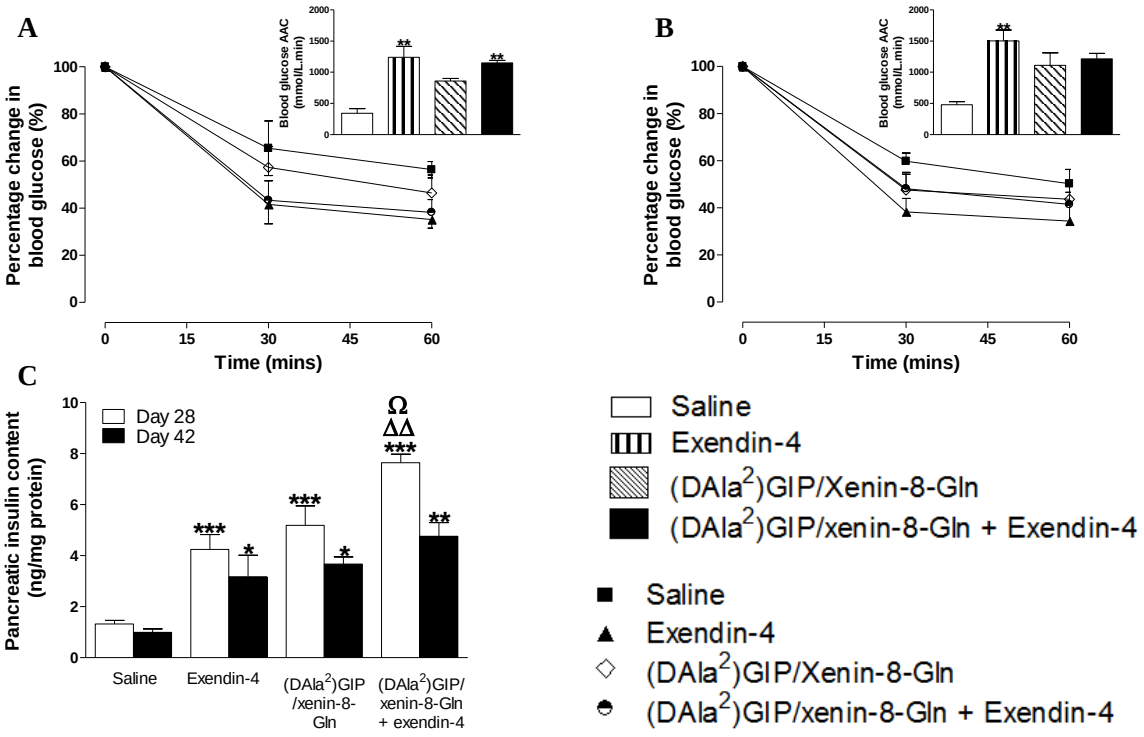
(A,B) Parameters were measured after 28 days twice daily treatment with (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides, or (C,D) 14 days following cessation of all treatments. (A-D) Glucose (18 mmol/kg bw) was administered i.p. at t = 0 min in overnight fasted mice. Glucose and insulin AUC values for 0–120 min post injection are also shown. Black squares and white bars, saline control; black triangles and black vertical line bars, exendin-4; white diamonds and diagonal line bars, (DAla^2)GIP/xenin-8-Gln; black/white circles and black bars, (DAla^2)GIP/xenin-8-Gln + exendin-4. All values are mean ± SEM for six mice. *P<0.05 and **P<0.01 compared with HFF saline controls.

Figure 3.4. Effects of (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides on acute metabolic response to respective peptide treatments in HFF mice.



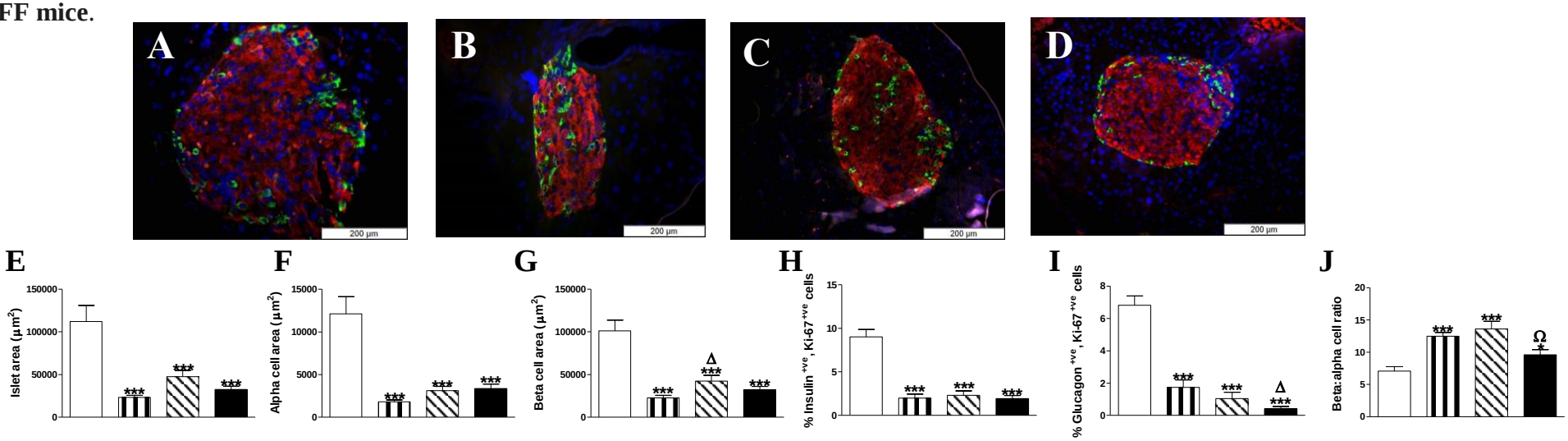
(A,B) Parameters were measured after 28 days twice daily treatment with (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides, or (C,D) 14 days following cessation of all treatments. (A-D) Glucose (18 mmol/kg bw) in combination with acute i.p. injection of the same respective treatment intervention (saline, (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides was administered at t = 0 min in overnight fasted mice. Glucose and insulin AUC values for 0–120 min post injection are also shown. Black squares and white bars, saline control; black triangles and black vertical line bars, exendin-4; white diamonds and diagonal line bars, (DAla^2)GIP/xenin-8-Gln; black/white circles and black bars, (DAla^2)GIP/xenin-8-Gln + exendin-4. Values are mean ± SEM for six mice. *P<0.05, **P<0.01 and ***P<0.001 compared with HFF saline controls. $^{\triangle}$P<0.05, $^{\triangle\triangle}$P<0.01 and $^{\triangle\triangle\triangle}$P<0.001 compared with exendin-4 alone. $^{\Omega}$P<0.05, $^{\Omega\Omega}$P<0.01 and $^{\Omega\Omega\Omega}$P<0.001 compared with (DAla^2)GIP/xenin-8-Gln alone.

Figure 3.5. Effects of (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides on insulin sensitivity and pancreatic insulin content in HFF mice.



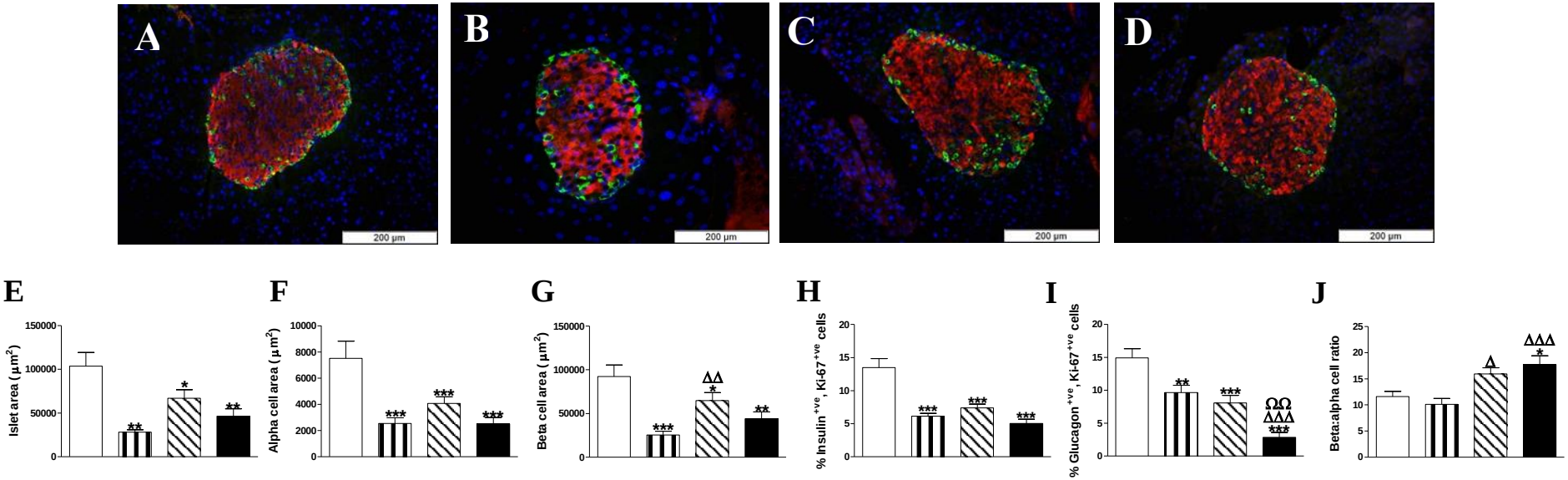
(A) Parameters were measured after 28 days twice daily treatment with (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides, or (B) 14 days following cessation of all treatments. (A,B) Insulin (25 U/kg bw) was administered i.p. at t = 0 min in non-fasted mice. Glucose AAC values for 0–60 min post injection are also shown. (C) Pancreatic insulin content was measured by RIA following acid-ethanol extraction. Values are mean ± SEM for six mice. *P<0.05, **P<0.01 and ***P<0.001 compared with HFF saline controls. $^{\Delta\Delta}$P<0.01 compared with exendin-4 alone. $^{\Omega}$P<0.05 compared with (DAla^2)GIP/xenin-8-Gln alone.

Figure 3.6. Effects of (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides on day 28 on pancreatic islet architecture in HFF mice.



(A-H) Parameters were measured after 28 days twice daily treatment with (DAla^2)GIP/xenin-8-Gln (25 nmol/kg bw), exendin-4 (25 nmol/kg bw) or a combination of both peptides. (A-D) Representative images (20X) of islets showing insulin (red) and glucagon (green) immunoreactivity from each treatment group. (E) Islet (F) alpha and (G) beta cell areas, as well as (H) insulin and (I) glucagon positive Ki-67 cells and (J) beta:alpha area cell area ratio, were measured using Cell^F image analysis software. White bars, saline control; black vertical line bars, exendin-4; diagonal line bars, (DAla^2)GIP/xenin-8-Gln; black bars, (DAla^2)GIP/xenin-8-Gln + exendin-4. Scale bar, 200 µm. All values are mean ± SEM for six mice. *P<0.05 and ***P<0.001 compared with HFF saline controls. $^{\triangle}$P<0.05 compared with exendin-4 alone. $^{\Omega}$P<0.05 compared with (DAla2)GIP/xenin-8-Gln alone.

Figure 3.7. Effects of (DAla^2)GIP/xenin-8-Gln, exendin-4 and a combination of both peptides 14 days following cessation of all treatment on pancreatic islet architecture in HFF mice.



(A-H) Parameters were measured 14 days following cessation of all treatments. (A-D) Representative images (20X) of islets showing insulin (red) and glucagon (green) immunoreactivity from each treatment group. (E) Islet (F) alpha and (G) beta cell areas, as well as (H) insulin and (I) glucagon positive Ki-67 cells and (J) beta:alpha area cell area ratio, were measured using Cell^F image analysis software. White bars, saline control; black vertical line bars, exendin-4; diagonal line bars, (DAla^2)GIP/xenin-8-Gln; black bars, (DAla^2)GIP/xenin-8-Gln + exendin-4. Scale bar, 200 µm. All values are mean ± SEM for six mice. *P<0.05, **P<0.01 and ***P<0.001 compared with HFF saline controls. $^{\triangle}$P<0.05, $^{\triangle\triangle}$P<0.01 and $^{\triangle\triangle\triangle}$P<0.001 compared with exendin-4 alone. $^{\Omega\Omega}$P<0.01 compared with (DAla^2)GIP/xenin-8-Gln alone.

Chapter 4

Assessing the acute biological actions and antidiabetic activity of novel xenin-25 C-terminal hexapeptides on pancreatic islet function and metabolism

4.1 SUMMARY

The K-cell derived peptide hormone, xenin-25, is known to undergo rapid metabolism following secretion into the bloodstream. Early studies revealed bioactivity of a C-terminal hexapeptide fragment peptide of xenin-25, namely xenin-6, which were enhanced through introduction of a reduced peptide bond between the first two N-terminal residues, to yield Ψ-xenin-6. The present study was undertaken to further assess biological actions and antidiabetic properties of Ψ-xenin-6, as well as characterisation of four additional novel xenin hexapeptides with Gln substitutions. Introduction of a reduced peptide bond in Ψ-xenin-6, unlike in the other four hexapeptides, resulted in completely resistant peptide to murine enzyme degradation *in vitro*. In BRIN-BD11 and INS-1 cells, as well as isolated murine islets, insulin-releasing actions of Ψ-xenin-6 were superior to xenin-6 and all other modified hexapeptides. Ψ-xenin-6 also prominently enhanced ($P<0.001$) the insulinotropic actions of GIP, with equal or increased potency compared to xenin-25. In α-TC1.9 cells, all hexapeptides had no impact on glucagon secretion, although, xenin-6 and xenin-6-NH_2, but not xenin-6-Gln, xenin-6-Gln-NH_2 or Ψ-xenin-6, partially reversed ($P<0.001$) the glucagonotropic action of GIP. However, in isolated murine islets, only Ψ-xenin-6 significantly ($P<0.05$) increased glucagon secretion, and further enhanced ($P<0.001$) the glucagonotropic actions of GIP, when compared to respective glucose control. Further *in vitro* investigations revealed that, similar to GLP-1, Ψ-xenin-6 significantly augmented proliferation of human and rodent clonal beta cells, whilst fully protecting against cytokine-induced beta-cell cytotoxicity. Xenin-6-Gln and xenin-6-Gln-NH_2 also augmented ($P<0.001$) proliferation of rodent clonal beta cells, with xenin-6-Gln having a significant ($P<0.05$) effect in 1.1B4 cells at the same concentration. All peptides exhibited protective mechanisms against cytokine-induced

beta cell cytotoxicity. When administered to mice in combination with glucose, all test peptides, barring xenin-6-Gln and xenin-6-NH_2, significantly ($P<0.05$ - $P<0.01$) reduced glucose levels. Ψ-xenin-6 had a duration of biological action beyond 8 hours. Ψ-xenin-6 also significantly augmented the glucose-lowering action of GIP *in vivo*. In overnight fasted mice, only Ψ-xenin-6 exhibited satiety actions at both 25 and 250 nmol/kg. Overall, this data demonstrates that Ψ-xenin-6 is a metabolically stable C-terminal fragment analogue of xenin-25, with an action profile that merits further study as a potential antidiabetic compound.

4.2 INTRODUCTION

Xenin-25, a 25 amino acid gut hormone, co-secreted from enteroendocrine K-cells along with GIP, has previously been shown to decrease gastrointestinal motility and modulate energy balance [Cline *et al*. 2007; Leckstrom *et al*. 2009; Kerbel *et al*. 2018]. Further studies also suggest that xenin-25 plays a role in regulating islet function and survival [Silvestre *et al*. 2003; Taylor *et al*. 2010; Martin *et al*. 2012; 2014; Gault *et al*. 2015; Parthsarathy *et al*. 2016; Khan *et al*. 2017a]. In this regard, others have also suggested therapeutic potential of xenin-25 for diabetes [Craig *et al*. 2018], as xenin-25 has also been shown to potentiate the insulinotropic actions of GIP [Wice *et al*. 2010, 2012; Martin *et al*. 2012; Gault *et al*. 2015]. Notwithstanding this, the evident physiological importance of xenin-25 has been somewhat overlooked to date [Maryanovich *et al*. 2018; Craig *et al*. 2019]. In a similar fashion, the consequence of xenin-25 enzymatic degradation, and biological relevance of related circulating xenin metabolites, is not fully understood. Thus, numerous regulatory gut-derived peptides possess dramatically altered biological action profile following enzymatic degradation

in the circulation [Deacon 2004; Mayorov *et al*. 2008; Lafferty *et al*. 2018; Craig *et al*. 2019].

A number of C-terminally truncated metabolites of xenin-25 have already been characterised including, xenin 9-25, xenin 11-25, xenin 14-25 and xenin 18-25 [Martin *et al*. 2014]. However, the biological significance of xenin 9-25, xenin 11-25 and xenin 14-25 is largely unknown [Martin *et al*. 2014]. Interestingly, xenin 18-25, also referred to as xenin-8, has been shown to recapitulate the effects of the parent peptide at the level of the endocrine pancreas [Silvestre *et al*. 2003; Martin *et al*. 2014; 2016; Craig *et al*. 2018] and duodenum [Kaji *et al*. 2017], as well as potentiating the biological actions of GIP [Martin *et al*. 2014; 2016]. This is particularly encouraging in terms of prospective therapeutic application of xenin, since smaller peptide size will lead to reduced production costs, promote simpler drug formulation, which together will dramatically increase overall commercial attractiveness. Following on from this, two very early studies in the xenin field had demonstrated bioactivity of a C-terminal hexapeptide of xenin-25, xenin-20-25 or also known as xenin-6 [Feurle *et al*. 1996, 2003]. However, full investigations into the potential of xenin-6 are lacking. Encouragingly, enzymatic stability and biological efficacy of this xenin hexapeptide was shown to be substantially enhanced through introduction of a reduced pseudopeptide bond (CH_2NH) between Lys^{20} and Arg^{21} amino acid residues, to yield a Psi (Ψ) pseudopeptide analogue named Ψ-xenin-6 [Feurle *et al*. 2003]. However, further application of these initial exciting observations with Ψ-xenin-6 have been missing until now.

In chapter 3, the persistent antidiabetic effects of xenin-8-Gln were characterised as part of a hybrid peptide in combination with an already established antidiabetic drug type, exendin-4. However, shorter analogues may be more therapeutically attractive, due to cheaper and easier synthesis, and may also enable a non-injectable peptide drug delivery [Novakovic *et al.* 2013; Park *et al*. 2015], which would improve patient compliance when moving towards the clinic. Therefore, the present study aimed to further characterise Ψ-xenin-6, along with novel Gln- substituted xenin hexapeptides, with a view to potential therapeutic application in the field of diabetes. The peptides assessed in this chapter were xenin-6, xenin-6-NH_2, xenin-6-Gln, xenin-6-Gln-NH_2 and Ψ-xenin-6. Initially susceptibility to plasma degradation was assessed, followed by *in vitro* and *ex vivo* assessment on insulin and glucagon secretion from BRIN-BD11, INS-1 and α-TC1.9 cells as well as isolated mouse islets, as appropriate, for all novel hexapeptides. In addition, effects on rodent and human beta cell proliferation and protection against cytokine-induced apoptosis were also assessed in BRIN-BD11 and 1.1B4 cell lines. Finally, impact of each peptide on glucose homeostasis, insulin secretion and satiety were examined in mice. The results revealed that Ψ-xenin-6 was the most potent hexapeptide investigated. Therefore, taken together, the results support further investigation of the potential therapeutic promise of Ψ-xenin-6 for diabetes.

4.3 MATERIALS AND METHODS

4.3.1 Peptides

All peptides were obtained as previously described in Section 2.2. Peptides were confirmed pure by HPLC (Section 2.3.1) and subsequently characterised using MALDI-TOF MS (Section 2.3.2).

4.3.2 Assessment of plasma degradation of peptides

Effect of murine plasma on peptide stability was assessed as described previously in Section 2.4.1.

4.3.3 *In vitro* and *ex vivo* insulin secretion

In vitro insulin secretory studies were performed using BRIN-BD11 and INS-1 cells as described previously in Section 2.6.1. In a separate set of experiments, BRIN-BD11 cells were incubated at 5.6 mmol/l glucose with test peptides in the presence of GIP (10^{-6} mol/l) for 20 minutes, as outlined in Section 2.6.1, and insulin measured by RIA as described in Section 2.11.2. In a final set of experiments, BRIN-BD11 cells were cultured at 22.2 mmol/l glucose and insulinotropic effects of GIP, xenin-6 and Ψ-xenin-6 determined, as described in Section 2.6.1. Effects of xenin hexapeptides on *ex vivo* insulin secretion were examined using isolated mouse islets (Section 2.10.2).

4.3.4 Assessment of toxicity of peptides

Toxicity of test peptides (10^{-6} mol/l, 20 minutes) was determined by assessing levels of LDH release in BRIN-BD11 cells, as described in Section 2.7.1.

4.3.5 *In vitro* and *ex vivo* glucagon secretion

In vitro glucagon secretory studies were performed using α-TC1.9 cells (Section 2.8.1). *Ex vivo* glucagon secretory effects were examined using isolated mouse islets as described in Section 2.10.3. Glucagon concentrations were measured by chemiluminescent ELISA (Section 2.12.1).

4.3.6 *In vitro* beta cell proliferation and apoptosis

In vitro effects of peptides on proliferation and apoptosis were performed using BRIN-BD11 and 1.1B4 cells as described previously in Section 2.9.1.

4.3.7 Animals

Acute and persistent dose dependent studies were carried out in lean mice. All animals were maintained as described previously in Section 2.13.1.

4.3.8 Acute *in vivo* effects of peptides on glucose-lowering, insulinotropic activity and food intake in lean mice

For food intake, studies were carried out as described in Section 2.14.1. Effects on glucose homeostasis and insulin secretory activity of test peptides alone or in combination with GIP were carried out as described in Section 2.14.2. Persistent peptide effects were assessed as outlined in Section 2.14.3.

4.3.9 Biochemical analysis

Blood samples were collected as previously described in Section 2.15 at the time points indicated in the Figures. Blood glucose and plasma insulin were assayed as described in Sections 2.11.2 and 2.15.

4.3.10 Statistical analysis

Statistical analysis was completed using GraphPad PRISM (Version 5) as described in Section 2.18.

4.4 RESULTS

4.4.1 Xenin hexapeptide characterisation and plasma enzymatic stability

In-house characterisation confirmed successful synthesis of test peptides using HPLC and MALDI-TOF MS (Table 4.1). Experimental and theoretical mass of each peptide was closely related, confirming successful synthesis of peptides (Table 4.1). In addition, Ψ-xenin-6 was completely resistant to plasma degradation for up to 6 h, whereas native xenin-6, xenin-6-NH_2, xenin-6-Gln and xenin-6-Gln-NH_2 had an estimated *in vitro* half-life of less than 2 h (Table 4.2).

4.4.2 Insulin secretory actions of novel xenin hexapeptides

In BRIN-BD11 cells at 1.1 mmol/l glucose, only xenin-6-Gln elicited significant ($P<0.05$ - $P<0.001$) augmentations of insulin secretion when compared to glucose control, with xenin-6-Gln-NH_2 displaying significant ($P<0.05$) insulinotropic effects at 10^{-6} mol/l concentrations (Figure 4.1A). All xenin hexapeptides displayed significant ($P<0.05$ - $P<0.001$) dose-dependent insulin secretory actions at 5.6 mmol/l glucose when compared to respective glucose controls (Figure 4.1B). Interestingly, at 5.6 mmol/l glucose, xenin 6-Gln and Ψ-xenin-6 exhibited superior ($P<0.05$ - $P<0.001$) insulinotropic effects than native xenin-6 at 10^{-12}, 10^{-10} and 10^{-6} mol/l peptide concentrations, with xenin-6-Gln-NH_2 exhibiting significant ($P<0.01$ - $P<0.001$) effects at 10^{-12} and 10^{-10} mol/l (Figure 4.1B). At 16.7 mmol/l glucose, Ψ-xenin-6 displayed significant ($P<0.01$ - $P<0.001$) insulinotropic effects when compared to respective glucose controls at 10^{-8} and 10^{-6} mol/l concentrations, with superior ($P<0.05$) effects compared to xenin-6 at 10^{-6} mol/l concentrations. All other peptides failed to elicit any significant insulinotropic increase (Figure. 4.1C). When xenin peptides were co-incubated with GIP in BRIN-BD11 cells, all hexapeptides, except

xenin-6-NH_2, significantly ($P<0.05$ - $P<0.001$) augmented the insulinotropic actions of GIP at 10^{-8} and 10^{-6} mol/l concentrations (Figure 4.2). In this regard, Ψ-xenin-6 exhibited superior ($P<0.01$ - $P<0.001$) GIP-potentiating efficacy than all xenin-6 peptides at all concentrations examined (Figure 4.2). The potent insulin-releasing actions of Ψ-xenin-6, were again superior over all other peptides tested, as confirmed in isolated murine islets (Figure 4.3).

None of the xenin peptides compromised BRIN-BD11 cell plasma membrane integrity, as displayed by lack of effects on LDH levels (Figure 4.4A). Under glucotoxic culture conditions, the insulin secretory responses of GIP, xenin-6 and Ψ-xenin-6 were significantly ($P<0.05$ - $P<0.01$) impaired (Figure 4.4B) Interestingly, when xenin-6 was co-incubated with GIP, the insulinotropic response was not significantly different in BRIN BD11 cells cultured under normal or glucotoxic culture conditions (Figure 4.4B). In contrast, although co-incubation with Ψ-xenin-6 and GIP under glucotoxic conditions augmented insulin secretion to a higher degree than co-incubation with xenin-6 and GIP, insulin secretion was reduced ($P<0.01$) when compared to control cultures. In INS-1 cells, only Ψ-xenin-6 exhibited significant ($P<0.001$) insulinotropic effects at 10^{-8} and 10^{-6} mol/l, with xenin-6, xenin-6-NH_2 and xenin-6-Gln-NH_2 eliciting significant ($P<0.01$ - $P<0.001$) insulin secretory effects at 10^{-6} mol/l (Figure 4.5). Ψ-xenin-6 also exhibited superior insulinotropic effects compared to xenin-6 at 10^{-12}, 10^{-10} and 10^{-8} mol/l (Figure 4.5).

4.4.3 Glucagon secretory actions of novel xenin hexapeptides

As expected, arginine and GIP augmented ($P<0.001$) glucagon secretion from α-TC1.9 cells (Figure 4.6A). Alone, none of the hexapeptides altered glucagon secretion

(Figure 4.6A). However, xenin-6 and xenin-6-NH_2 partially reversed the glucagonotropic actions of GIP, whereas xenin-6-Gln, xenin-6-Gln-NH_2 and Ψ-xenin-6 did not (Figure 4.6A). Ψ-xenin-6 significantly ($P<0.01$) augmented the glucagonotropic actions of GIP (Figure 4.6A). The observations on glucagon secretion following incubation of arginine, GIP, xenin-6, xenin-6-NH_2, xenin-6-Gln, xenin-6-Gln-NH_2 and Ψ-xenin-6 in α-TC1.9 cells were similar in isolated murine islets (Figure 4.6B). All peptides except Ψ-xenin-6 partially reversed the glucagonotropic actions of GIP. In addition, whilst the ability of xenin-6 to reverse GIP-induced elevations of glucagon secretion was again apparent, this failed to reach significance in mouse islets (Figure 4.6B).

4.4.4 Effects of novel xenin hexapeptides on beta cell proliferation and protection against apoptosis

Similar to GLP-1, Ψ-xenin-6 significantly ($P<0.001$) augmented BRIN-BD11 and 1.1B4 beta cell proliferation at both 10^{-8} and 10^{-6} mol/l (Figure 4.7A,B). Xenin-6-Gln enhanced BRIN-BD11 and 1.1B4 beta cell proliferation at 10^{-6} mol/l, with xenin-25 and xenin-6-Gln-NH_2 only displaying beta cell proliferative effects in BRIN-BD11 cells at 10^{-6} mol/l (Figure 4.7A). Xenin-6 and xenin-6-NH_2 displayed no proliferative actions in BRIN-BD11 or 1.1B4 beta cells (Figure 4.7A,B). In relation to protection against cytokine-induced apoptosis, all test peptides reversed detrimental DNA damaging effects of co-incubation with cytokines in BRIN-BD11 cells, but only at the highest concentration employed (Figure 4.7C). In 1.1B4 beta cells, Ψ-xenin-6 offered full protection against cytokine-induced apoptosis at both 10^{-8} and 10^{-6} mol/l, with benefits of GLP-1, xenin-25, xenin-6, xenin-6-NH_2, xenin-6-Gln and xenin-6-Gln-NH_2 only observed at 10^{-6} mol/l (Figure 4.7D). Furthermore, in 1.1B4 cells Ψ-xenin-

6 (10^{-6} M) reduced ($P<0.05$) cytokine-induced elevations of TUNEL staining to below control levels (Figure 4.7D).

4.4.5 Acute effects of novel xenin hexapeptides on *in vivo* food intake

At a dose of 25 nmol/kg, only xenin-25 and Ψ-xenin-6 induced a significant ($P<0.01$ and $P<0.05$) reduction in food intake compared to saline controls (Figure 4.9A). All other treatments had no effect on re-feeding at a dose of 25 nmol/kg (Figure 4.9A). Interestingly, at a supraphysiological dose (250 nmol/kg), only Ψ-xenin-6 exhibited appetite suppressive actions ($P<0.05$), evident at both 30 and 180 min post-injection (Figure 4.9B), with xenin-6-Gln-NH_2 displaying suppressive effects 30 min post injection ($P<0.05$; Figure 4.9B). All other xenin hexapeptides, including native xenin-6, did not exhibit any significant appetite suppressive effects at either of the doses employed (Figure 4.9A,B). When in combination with exendin-4, all hexapeptides assessed significantly reduced food intake compared to saline (Figure 4.10).

4.4.6 Acute glucose lowering and insulin releasing effects of novel xenin hexapeptides in lean mice

Administration of xenin-6, xenin-6-Gln-NH_2 and Ψ-xenin-6, concomitantly with glucose resulted in significantly ($P<0.05$ - $P<0.01$) decreased overall 0-105 min AUC glucose when compared to glucose alone (Figure 4.11B). Treatment with GIP, xenin-6, xenin-6-Gln-NH_2 and Ψ-xenin-6 elevated ($P<0.05$ - $P<0.001$) corresponding 0-105 min AUC insulin concentrations (Figure 4.11D). When administered in combination with GIP, there was a significant ($P<0.01$ - $P<0.001$) reduction in glucose levels at 30 and 60 min post-injection when compared to glucose alone (Figure 4.12A). Reductions in overall AUC values when compared to GIP alone were only observed

when GIP was combined with Ψ-xenin-6 ($P<0.05$), but not with the other xenin-6 peptides assessed (Figure 4.12B). All treatments, barring xenin-6-Gln, elevated ($P<0.05$ - $P<0.001$) corresponding 0-105 min AUC insulin concentrations, when compared to glucose control (Figure 4.12D). Overall AUC insulin levels demonstrated that xenin-6 and Ψ-xenin-6 augmented the insulinotropic actions of GIP (Figure 4.12D).

4.4.7 Persistent glucose lowering and insulin releasing effects of novel xenin hexapeptides in lean mice

Administration of xenin-6, xenin-6-Gln-NH_2 or Ψ-xenin-6 2 h prior to glucose load, resulted in significantly reduced individual ($P<0.05$ - $P<0.01$) and AUC overall ($P<0.05$ - $P<0.01$) glucose levels when compared to saline controls (Figure 4.13A,B). All other xenin hexapeptides were devoid of beneficial glucose-lowering effects at this time point (Figure 4.13A,B). Similar observations were made when xenin-6, xenin-6-Gln-NH_2 or Ψ-xenin-6 were injected 4 h before the glucose challenge (Figure 4.13C,D). When administered 8 h prior to the glucose load, xenin-6 reduced ($P<0.05$) individual glucose levels at 15 min post-injection, with Ψ-xenin-6 reducing ($P<0.05$) individual glucose levels at 30 min post-injection (Figure 4.13F). Both xenin-6 and Ψ-xenin-6 significantly ($P<0.05$) decreased overall AUC glucose values (Figure 4.13F). Xenin-6-Gln-NH_2 exhibited no beneficial effects when administered 8 h prior to glucose challenge. Both xenin-6 and Ψ-xenin-6 failed to elicit any significant glucose-lowering actions when delivered 12 h prior to a glucose challenge (Figure 4.13G,H).

4.5 DISCUSSION

Previous studies have established the antidiabetic potential of truncated metabolites of xenin-25 [Martin *et al*. 2014]. In chapter 3, the antidiabetic abilities of the truncated modified xenin-8 peptide, xenin-8-Gln, as a hybrid agent were explored. In this chapter, the aim was to further characterise truncated forms of xenin-25, namely xenin-6. Earlier reports established that chemical manipulation of the C-terminal hexapeptide of xenin-25, namely xenin-6, through introduction of a reduced pseudopeptide bond between Lys^{20} and Arg^{21}, yields an enzymatically stabilised peptide with enhanced biological activity [Feurle *et al*. 2003]. Similar improved metabolic stability and bioactivity has been observed in neurotensin, a peptide closely related to xenin [Feurle *et al*. 2002], following introduction of a reduced pseudopeptide bond [Lugrin *et al*. 1991]. These studies are in complete harmony with previous work from our laboratory, demonstrating enhanced stability and biological activity of an octapeptide C-terminal fragment of xenin-25 following rationally introduced amino-acid modifications [Irwin *et al*. 2012; Martin *et al*. 2016; Parthsarathy *et al*. 2016].

Consistent with this knowledge, in the present study xenin-25, xenin-6, xenin-6-NH_2, xenin-6-Gln, xenin-6-Gln-NH_2 and particularly Ψ-xenin-6 evoked clear increases of insulin secretion from BRIN-BD11 cells [Feurle *et al*. 1992; Taylor *et al*. 2010]. Indeed, all xenin hexapeptides had similar, or even enhanced efficacy when compared to the parent peptide, xenin-25. Notably, Ψ-xenin-6 and xenin-6-Gln were significantly more efficacious at 10^{-6} mol/l than xenin-6 and xenin-25 at 5.6 mmol/l glucose, which is interesting and may require further study. The insulinotropic potency of xenin peptides appeared to be somewhat reduced at higher glucose concentrations

in BRIN-BD11 cells, and this may be due to difficulties in assessing additive peptide effects in the face of increased insulin output by elevated glucose. Nevertheless, these observations corroborate that C-terminal hexapeptides of xenin-25 are capable of activating xenin related beta cell signalling pathways. Indeed, insulin secretory studies using functional isolated mouse islets fully confirmed this concept. At the beta cell level, notable GIP-potentiating actions of xenin-25 are recognised [Wice *et al*. 2010; 2012; Martin *et al*. 2012; Parthsarathy *et al*. 2016], which were reproduced in this study by all hexapeptides examined and to a significantly greater magnitude by Ψ-xenin-6. The effects of xenin-6 and Ψ-xenin-6 were further assessed in BRIN-BD11 cells cultured under glucotoxic conditions to mimic beta cell stress encountered in diabetes [Pathak *et al*. 2014].

As would be expected, there was an impairment of the insulin-releasing action of GIP and xenin-6 peptides. GIP-augmenting actions of the truncated xenin peptides were still observed under glucotoxic culture, especially in the case of xenin-6. Nonetheless, the most effective of the compounds assessed in terms of GIP-potentiation was Ψ-xenin-6, albeit with notably less efficacy than in normal beta cells. In addition, Ψ-xenin-6 was the only peptide examined that significantly enhanced the glucose-lowering action of GIP in mice. Thus, whilst the exact mechanism of xenin-induced GIP potentiation remains to be elucidated [Wice *et al*. 2010; Clemens *et al*. 1997; Mazella *et al*. 2012], these observations substantiate the idea that Ψ-xenin-6 possesses enhanced biological potency over xenin-6 and the other hexapeptides considered [Feurle *et al*. 2003]. It is also encouraging to note that the amino-acid modifications and the reduced pseudopeptide bond present within these novel hexapeptides had no detrimental impact in terms of beta cell cytotoxicity.

Based on previous observations of glucagonotropic actions of a C-terminal octapeptide fragment of xenin-25 [Silvestre *et al*. 2003], and evidence that GIP induces glucagon secretion under fasting glycaemic levels [Christensen *et al*. 2011], the aim was to further examine this concept with our modified xenin hexapeptides. Unlike GIP, all hexapeptides examined were devoid of glucagonotropic actions in α-TC1.9 cells and isolated rodent islets. This does slightly contrast with previous findings in rodent islets using xenin-8, although *in situ* perfusion as opposed to static incubations were employed for this earlier study [Silvestre *et al*. 2003]. In addition, the most prominent secretory effect of xenin-8 was noted to be potentiation of arginine- and carbachol-induced glucagon elevations [Silvestre *et al*. 2003]. Given this, and the notable interactions between the gut hormones xenin and GIP [Wice *et al*. 2012; Martin *et al*. 2012; Chowdhury *et al*. 2013; Irwin and Flatt, 2015; Hasib *et al*. 2017], the impact of co-incubation with the xenin-6 fragment peptides and GIP was considered. Interestingly, xenin-6 and xenin-6-NH_2, but not xenin-6-Gln, xenin-6-Gln-NH_2 and Ψ-xenin-6, had a strong tendency to reverse GIP-mediated increases in glucagon release. This is interesting, considering both augmentation and blockade of glucagon receptor signalling has been advocated as being beneficial in diabetes [Pathak *et al*. 2015; McShane *et al*. 2016]. The difference in biological activity between these C-terminal hexapeptides examined is intriguing and presumably not related to the enhanced stability of Ψ-xenin-6 noted here and elsewhere [Feurle *et al*. 2002; Craig *et al*. 2019]. However, it could also relate to structure/function complexities between the numerous peptides, which would require further in-depth study. In humans, GIP infusion has been shown to prevent insulin-induced hypoglycaemia, therefore the overall impact of xenin on GIP-induced alterations of glucagon secretion is of significant interest [Christensen, 2016]. Indeed,

this may represent an important homeostatic metabolic mechanism of GIP [Christensen *et al*. 2011], where xenin may also play a key role, and could have particular relevance in a diabetic setting.

Further to this, previous studies with xenin-25 have clearly highlighted potential benefits at the level of the beta cell. As well as enhancing insulin release and potentiating GIP-induced insulin release [Taylor *et al*. 2010; Wice *et al*. 2010; Martin *et al*. 2012, 2014], the parent xenin peptide has been shown to promote beta cell growth and survival [Khan *et al*. 2017a]. Xenin-6-Gln-NH_2 exhibited similar or enhanced beta cell proliferative properties in BRIN-BD11 cells, with xenin-6-Gln displaying enhanced proliferative properties in both BRIN-BD11 and 1.1B4 beta cells. Promisingly, Ψ-xenin-6 had similar, or even enhanced, beta cell proliferative and survival beneficial effects in both rodent BRIN-BD11 and human 1.1B4 beta cells. This is encouraging given that T2DM is a disease characterised by beta cell loss [Halban *et al*. 2014], although the exact mechanisms involved in these xenin-induced beneficial effects still need to be clarified. Similar to previous reports [Mohan *et al*. 2018, 2019], baseline apoptosis rates were somewhat elevated in both beta cell lines, representing a particularly challenging environment. As such, it may have been interesting to assess the benefits of xenin hexapeptides on beta cell survival under less severe beta cell insults. Indeed, the aforementioned positive effects of Ψ-xenin-6 on glucose homeostasis, insulin release and GIP potentiation, coupled with an *in vivo* duration of approximately 8 hours in mice, further promote its potential therapeutic value for diabetes. The observation of enhanced glucose homeostatic actions of combined GIP and Ψ-xenin-6 administration in mice, despite no obvious augmentation of insulin secretion, likely reflects enhancement of the notable

extrapancreatic glucose-lowering effects of GIP and xenin [Irwin and Flatt, 2015; Craig *et al*. 2018].

Furthermore, xenin has been suggested to supress food intake [Craig *et al*. 2018] and delay gastric emptying rate [Kim and Mizuno 2010], which would also be complementary in the T2DM setting [Al-Goblan *et al*. 2014]. In our system, Ψ-xenin-6 induced small, but significant, appetite suppressive effects in mice at doses of 25 and 250 nmol/kg, whereas all other xenin-6 peptides assessed were ineffective. Notably, physiological circulating concentrations of xenin are not well defined, and local tissue production of xenin has been documented outside of the gut [Khan *et al*. 2017a]. In addition, previous studies demonstrating prominent efficacy of xenin peptides to inhibit feeding have largely employed intracerebroventricular administration [Alexiou *et al*. 1998; Leckstrom *et al*. 2009; Cooke *et al*. 2009; Bhavya *et al*. 2017; Kerbel *et al*. 2018], as opposed to peripheral application in the present study. This may suggest that efficient passage through the blood-brain barrier is critical for xenin-based peptides to positively modulate energy balance.

In conclusion, these data substantiate the notion that Ψ-xenin-6 is a stable, long-acting xenin analogue that retains the full biological action profile of the parent peptide. Benefits of Ψ-xenin-6 on islet cell function and survival, appetite suppression and GIP-potentiation, coupled with a prolonged half-life, emphasise prospective therapeutic potential of this peptide for diabetes, above all other hexapeptides examined. Future studies should therefore evaluate the preclinical utility of Ψ-xenin-6 in appropriate models of diabetes, both alone and in combination with established antidiabetic drugs, such as incretin enhancers.

Table 4.1 Amino acid sequence, HPLC retention times, MALDI-TOF MS and murine plasma stability of xenin peptides

Peptide	Amino acid sequence	Modification	Theoretical molecular mass (Da)	Experimental molecular mass (Da)	Retention time (min)	Percentage purity (%)	*In vitro* half-life (murine plasma; h)
Xenin-6	H-LYS-ARG-PRO-TRP-ILE-LEU-OH	None	812.2	812.0	16.8	98.9	< 2 h
Xenin-6-NH_2	H-LYS-ARG-PRO-TRP-ILE-LEU-NH_2	C-Terminal Amidation	811.0	811.0	16.1	96.3	< 2 h
Xenin-6-Gln	H-GLN-GLN-PRO-TRP-ILE-LEU-OH	Lys & Arg deletion (Lys^{20}, Arg^{21}) and substitution with Gln	783.9	785.5	18.7	96.1	< 2 h
Xenin-6-Gln-NH_2	H-GLN-GLN-PRO-TRP-ILE-LEU-NH_2	Lys & Arg deletion (Lys^{20}, Arg^{21}) and substitution with Gln + C-terminal Amidation	782.9	783.8	17.6	97.6	< 2 h
Ψ-xenin-6	H-LYS-ψ-ARG-PRO-TRP-ILE-LEU-OH	Reduced peptide bond (between the Lys^{20} and Arg^{21} amino acid residues)	798.1	798.5	16.6	97.0	> 6 h

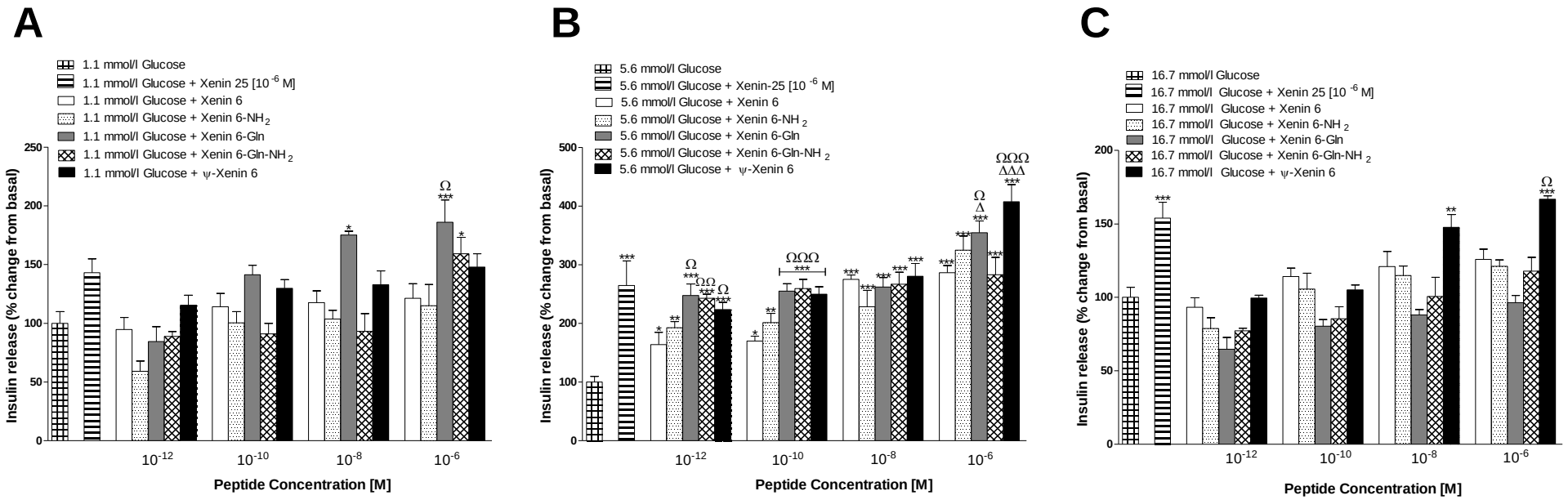
Peptides were confirmed pure using HPLC and retention times recorded using ChromQuest software. Molecular masses of peptides were confirmed using MALDI-TOF MS.

Table 4.2 Murine plasma stability of xenin hexapeptides at 0, 2, 4 and 6 hours.

Percentage Degradation (%)				
Peptide	**0h**	**2h**	**4h**	**6h**
Xenin-6	0	53.2	85.8	88.2
Xenin-6-NH_2	0	43.4	64.9	70.3
Xenin-6-Gln	0	54.7	65.5	66.6
Xenin-6-Gln-NH_2	0	45.9	57.4	64.3
Ψ-xenin-6	0	0	0	0

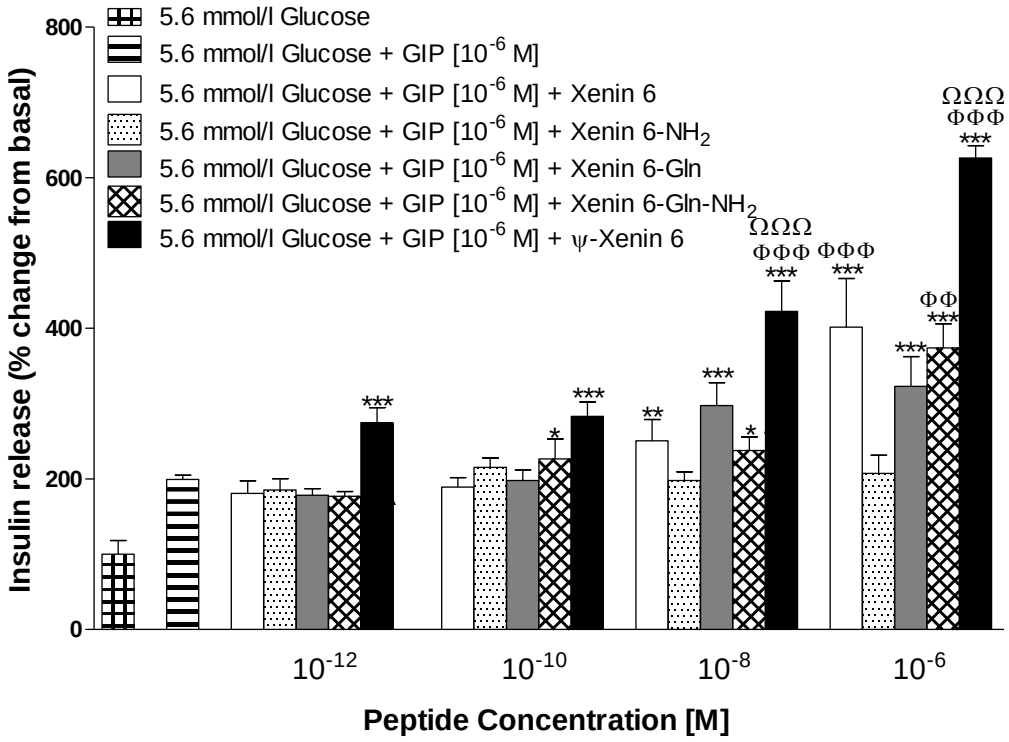
Peptide stability was assessed following 0, 2, 4 and 6 hr incubation with plasma. Degradation products were separated using HPLC and percentage degradation calculated from peak area.

Figure 4.1 Acute effects of xenin hexapeptides on insulin release from BRIN-BD11 cells



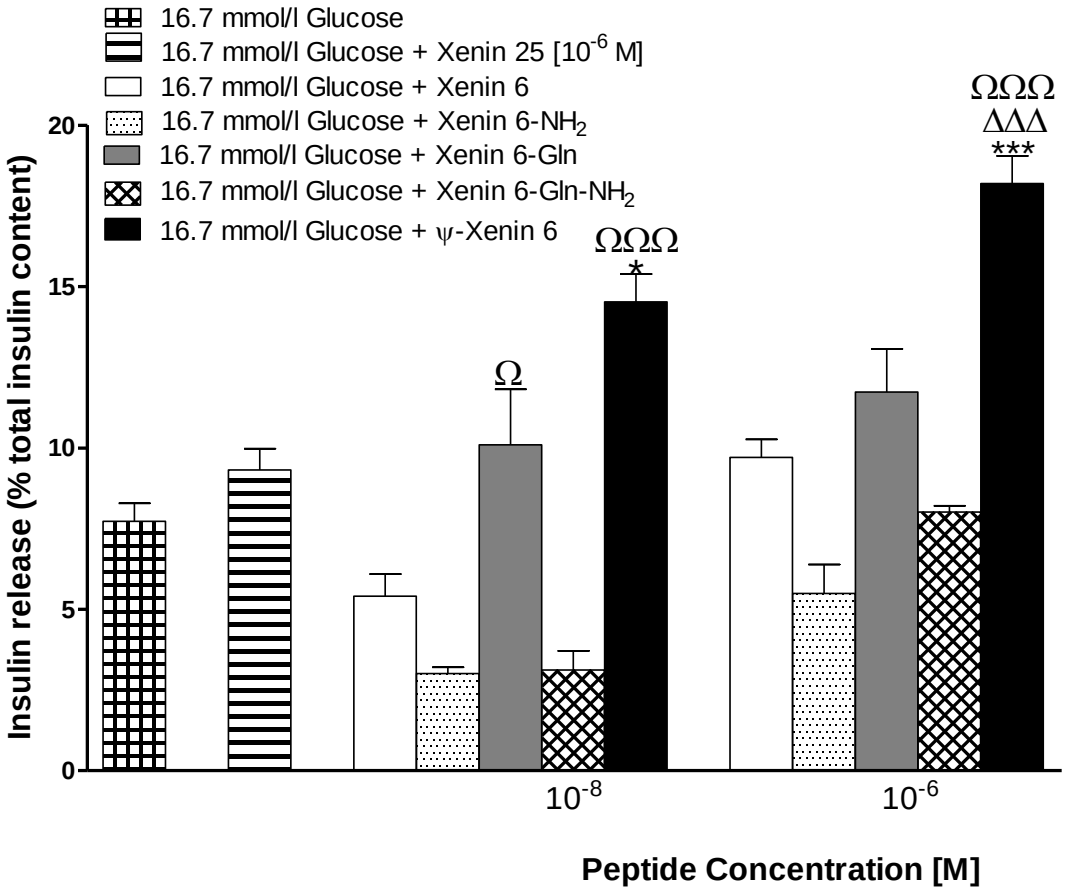
BRIN-BD11 cells were incubated (20 min) with a range of concentrations (10^{-12} to 10^{-6} mol/l) of test peptides in the presence of (A) 1.1, (B) 5.6 and (C) 16.7 mmol/l glucose, and insulin measured using RIA. Values represent means ± SEM (n=8). *P<0.05, **P<0.01 and ***P<0.001 compared to respective glucose controls. $^{\Delta}P< 0.05$ and $^{\Delta\Delta\Delta}P< 0.001$ compared to xenin-25. $^{\Omega}P< 0.05$, $^{\Omega\Omega}P< 0.01$, $^{\Omega\Omega\Omega}P< 0.001$ compared to xenin-6.

Figure 4.2 Acute effects of xenin hexapeptides in combination with GIP on insulin release from BRIN-BD11 cells



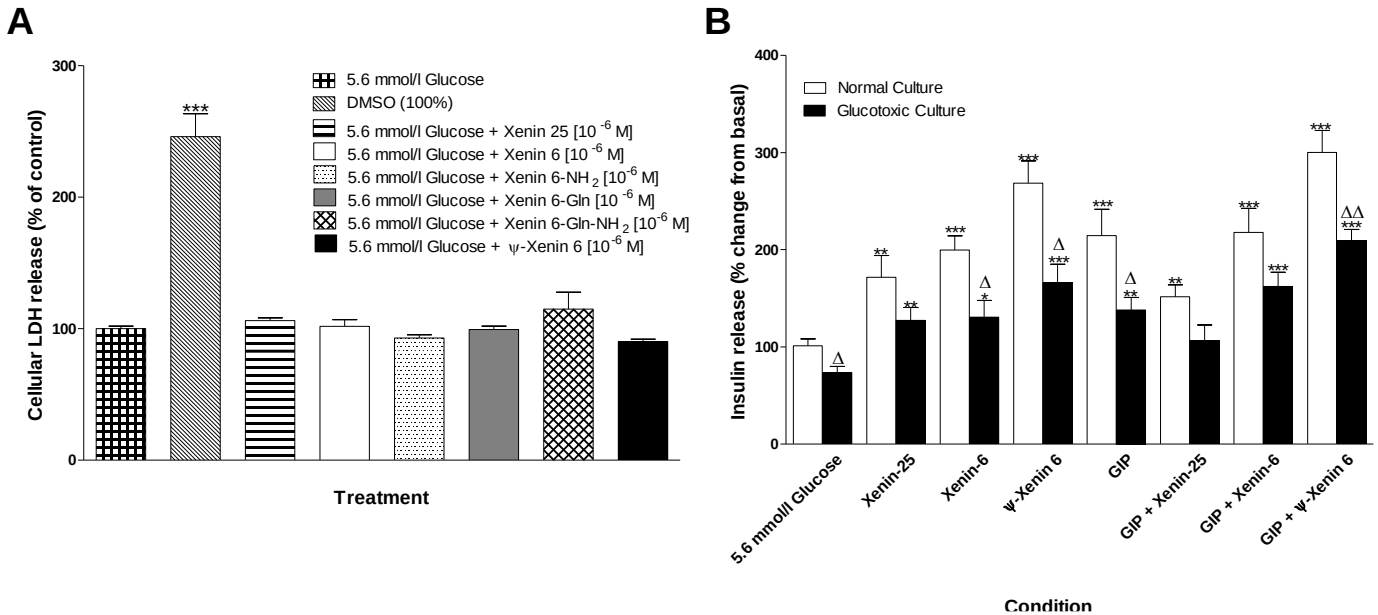
BRIN-BD11 were incubated (20 min) with a range of concentrations (10^{-12} to 10^{-6} mol/l) of test peptides in combination with GIP. Insulin was measured using RIA. Values represent means ± SEM (n=8). *P<0.05, **P<0.01 and ***P<0.001 compared to respective glucose control. $^{\Omega\Omega\Omega}$P<0.001 compared to xenin-6. $^{\Phi\Phi}$P<0.01, $^{\Phi\Phi\Phi}$P<0.001 compared to respective GIP control.

Figure 4.3 Acute effects of xenin hexapeptides on insulin release from isolated mouse islets



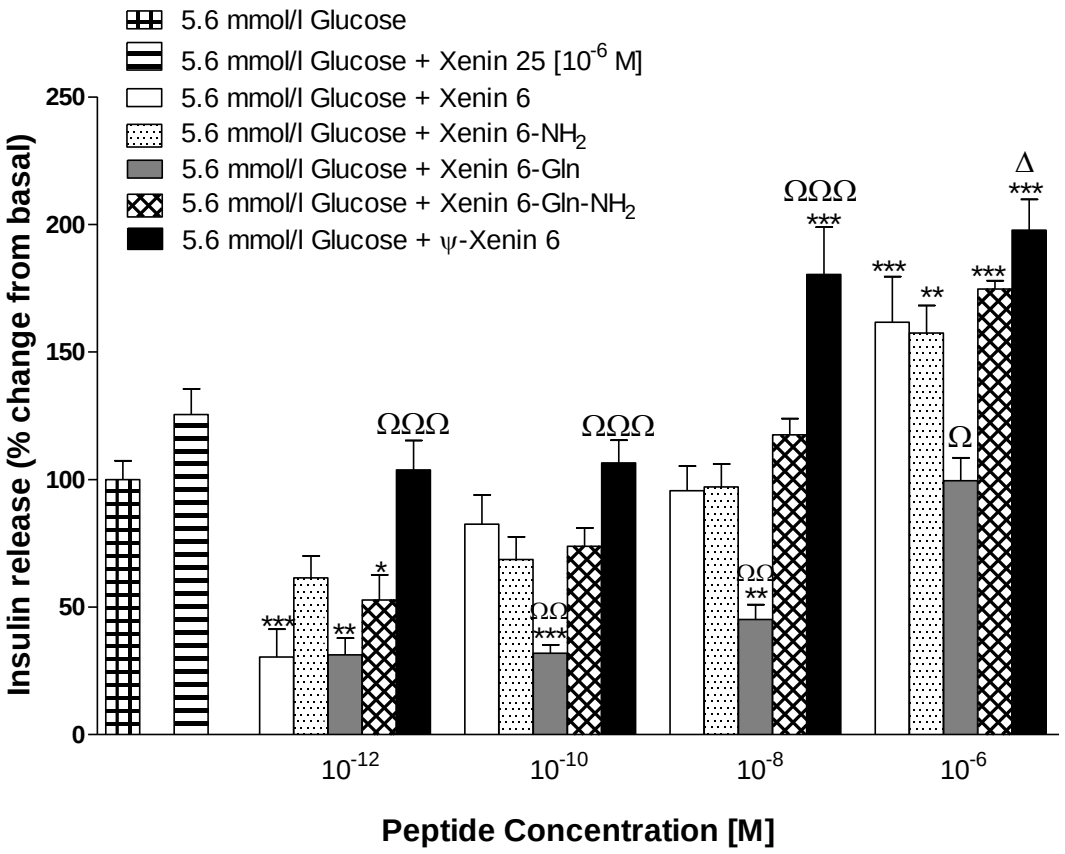
Insulin release was measured in isolated mouse islets incubated (60 min) with test peptides (10^{-8} and 10^{-6} M) at 16.7 mmol/l glucose. Insulin was measured using RIA. Values represent means ± SEM (n=8). *P<0.05 and ***P<0.001 compared to respective glucose controls. $^{\Delta\Delta\Delta}$P<0.001 compared to respective xenin-25 control. $^{\Omega}$P<0.05 and $^{\Omega\Omega\Omega}$P<0.001 compared to xenin-6.

Figure 4.4 Effects of xenin hexapeptides on LDH accumulation in BRIN-BD11 cells, as well as effects of xenin 6 and Ψ-xenin-6 on insulin release under glucotoxic conditions from BRIN-BD11 cells



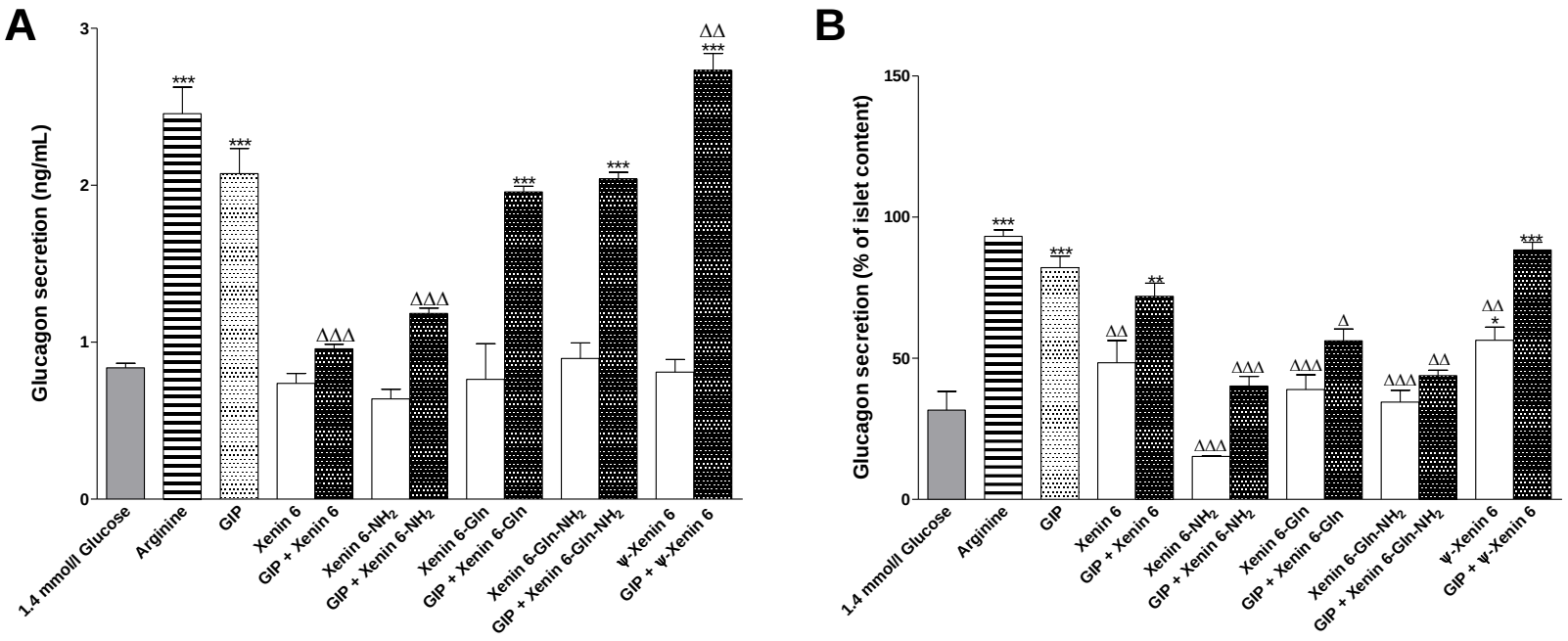
LDH accumulation was assessed in BRIN-BD11 cells following a 20 min incubation with 10^{-6} mol/l test peptides. BRIN-BD11 cells were cultured under glucotoxic (22.2 mmol/l glucose) culture conditions for 48 h and then incubated (20 min) with (10^{-6} mol/l) GIP, xenin-6 and Ψ-xenin-6 alone or in combination, as appropriate, in the presence of 5.6 mmol/l glucose. Insulin was measured using RIA. Values represent means ± SEM (A n=8; B n=4). *P<0.05, **P<0.01 and ***P<0.001 compared to respective glucose control. $^{\Delta}$P< 0.05 and $^{\Delta\Delta}$P< 0.01 compared to same concentration of respective peptide treatment under normal culture conditions.

Figure 4.5 Acute effects of xenin hexapeptides on insulin release from INS-1 cells



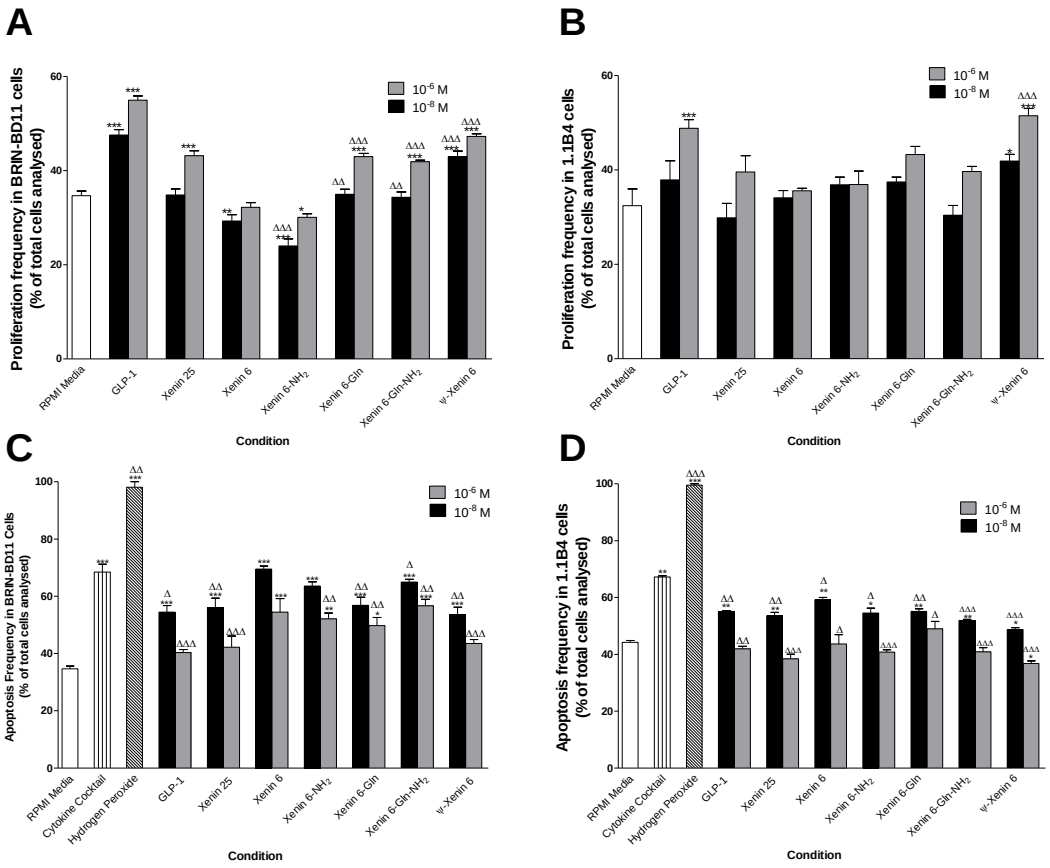
INS-1 cells were incubated (20 min) with a range of concentrations (10^{-12} to 10^{-6} mol/l) of test peptides in the presence of 5.6 mmol/l glucose, with insulin release measured using RIA. Values represent means ± SEM (n=8). *P<0.05, **P<0.01 and ***P<0.001 compared to respective glucose controls. $^{\Delta}$P< 0.05 compared to xenin-25. $^{\Omega}$P< 0.05, $^{\Omega\Omega}$P< 0.01 and $^{\Omega\Omega\Omega}$P< 0.001 compared to xenin-6.

Figure 4.6 Effects of xenin hexapeptides alone or in combination with GIP on glucagon secretion from (A) α-TC1.9 cells and (B) isolated mouse islets



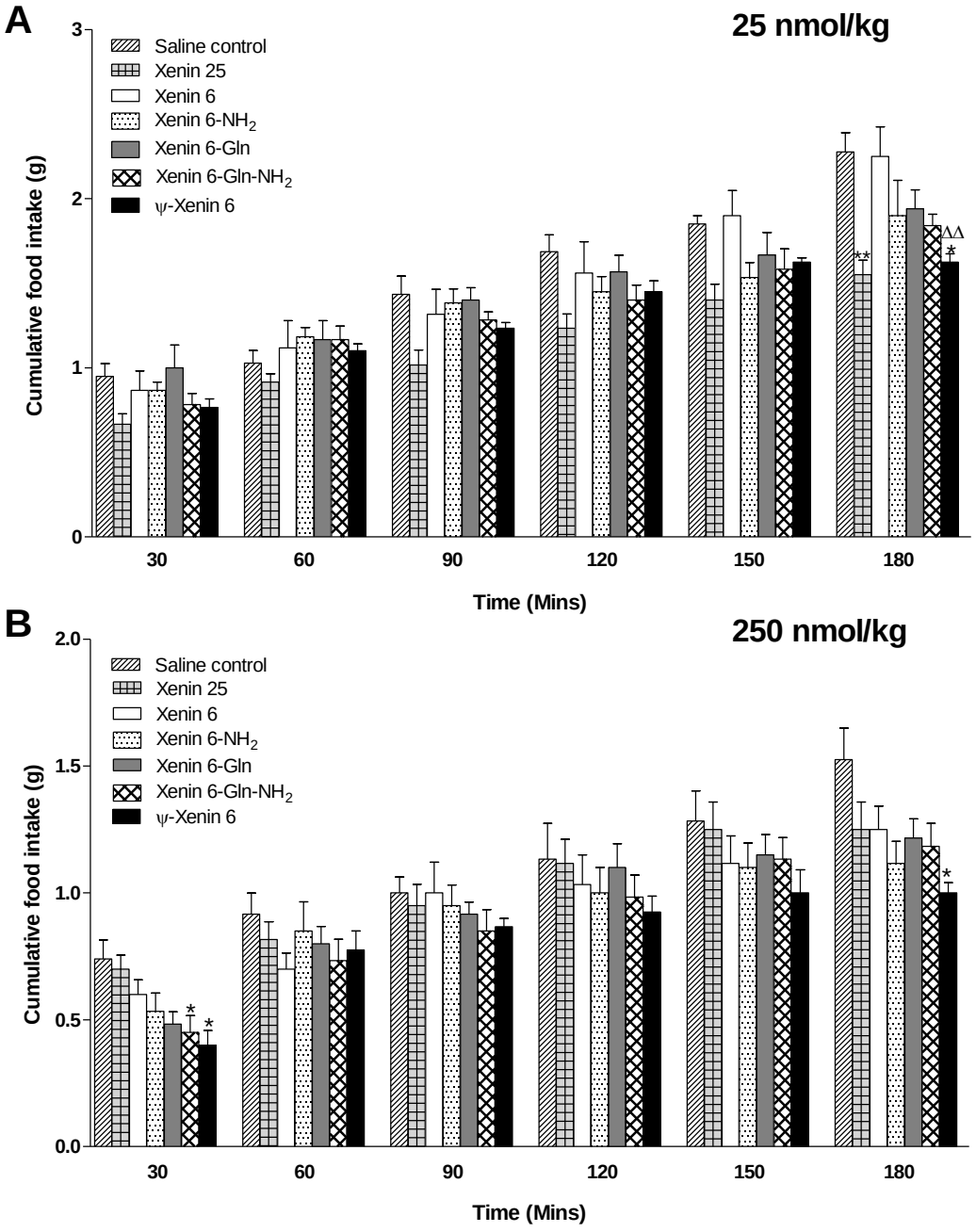
Effects of test peptides alone (10^{-6} mol/l) or in combination with GIP (10^{-6} mol/l) on glucagon secretion from (A) α-TC1.9 cells (120 min incubation) and (B) isolated mouse islets (60 min incubation) at 1.4 mmol/l glucose. Glucagon was measured using ELISA. Values represent means ± SEM (n=4). *P<0.05 and ***P<0.001 compared to 1.4 mmol/l glucose control. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to respective GIP control.

Figure 4.7 Effect xenin hexapeptides on proliferation and protection against apoptosis in rodent BRIN-BD11 and human 1.1B4 beta cells.



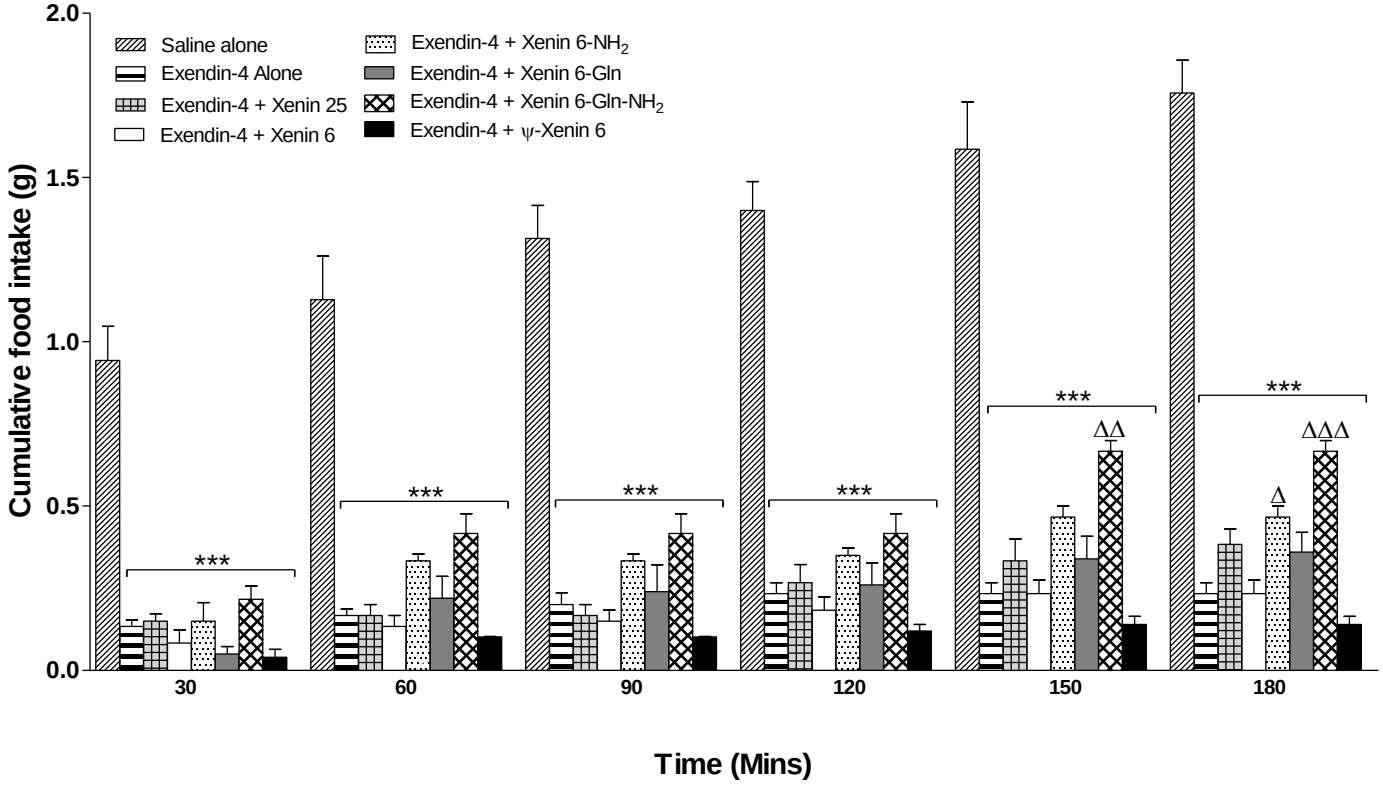
(A) BRIN-BD11 and (B) 1.1B4 beta-cells were incubated overnight (18 hours) with GLP-1, xenin-25, xenin-6, xenin-6-NH_2, xenin-6-Gln, xenin-6-Gln-NH_2 or Ψ-xenin-6 (each at 10^{-8} and 10^{-6} mol/l). Proliferation was measured using Ki-67 immunocytochemistry. (C,D) TUNEL positive apoptotic cells were assessed following 2 h exposure to a cytokine cocktail (IL-1β 100 U/mL, IFN-γ 20 U/mL, TNF-α 200 U/mL) with or without co-culture in the presence of GLP-1, xenin-25, xenin-6, xenin-6-NH_2, xenin-6-Gln, xenin-6-Gln-NH_2 or Ψ-xenin-6 (each at 10^{-8} and 10^{-6} mol/l) in (C) BRIN-BD11 and (D) 1.1B4 beta-cells. Values represent means ± SEM (n=4). For proliferation; *P<0.05, **P<0.01 and ***P<0.001 compared to respective media control. $^{\Delta\Delta}P$<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to xenin-6. For apoptosis; *P<0.05, **P<0.01 and ***P<0.001 compared to respective media control. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to cytokine cocktail alone.

Figure 4.9 Effects of xenin hexapeptides on cumulative food intake in 18 hour fasted lean mice.



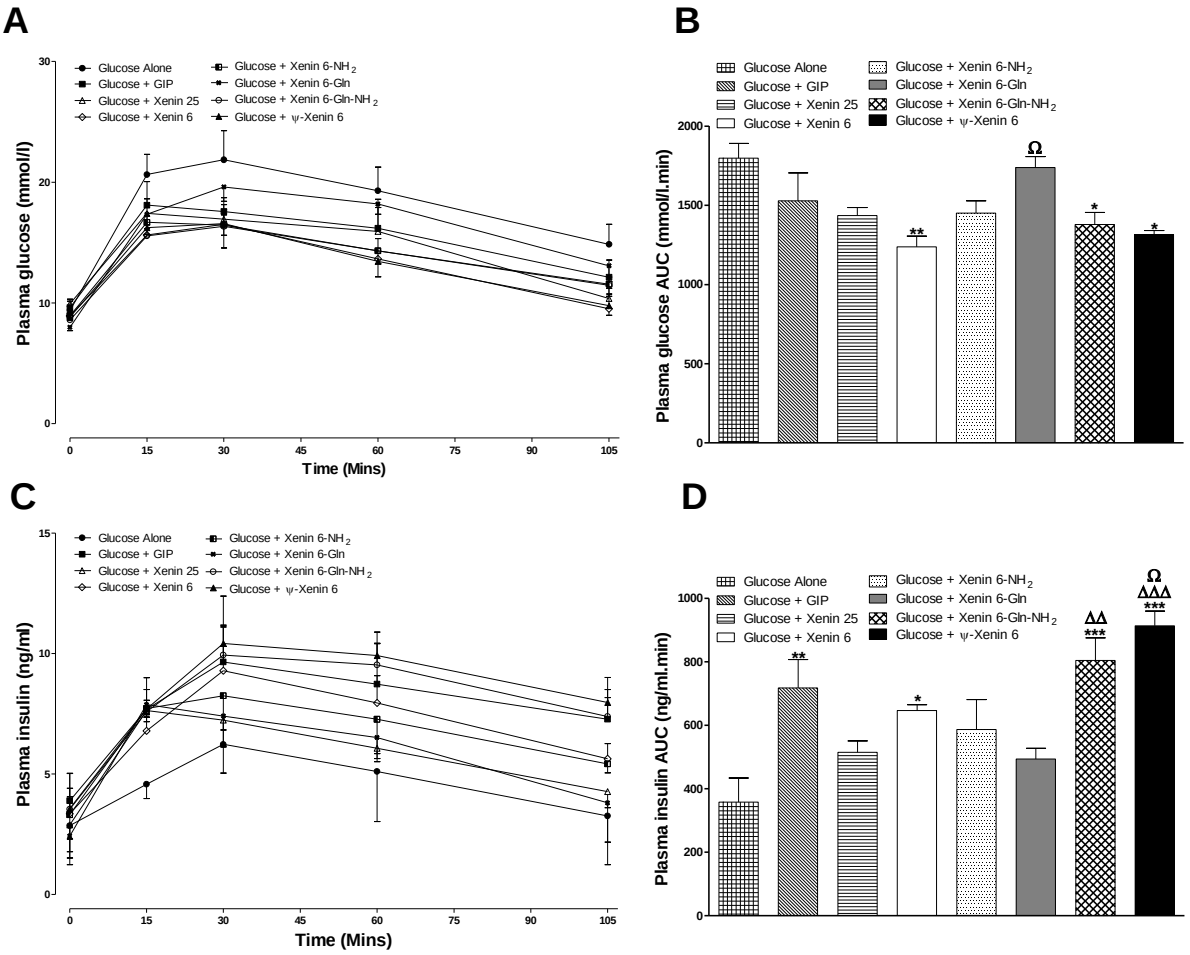
Cumulative food intake was measured in 18 h fasted mice at 30, 60, 90, 120, 150, and 180 min after i.p. injection of saline vehicle (0.9% w/v NaCl), xenin-25, xenin-6, xenin-6-NH_2, xenin-6-Gln, xenin-6-Gln-NH_2 or Ψ-xenin-6 at (A) 25 and (B) 250 nmol/kg bw. Values represent means ± SEM (n=8). *P<0.05 and **P<0.01 compared to respective saline control. $^{\Delta\Delta}$P<0.01 compared to xenin-6.

Figure 4.10 Effects of xenin hexapeptides in combination with exendin-4 on cumulative food intake in 18 hour fasted lean mice.



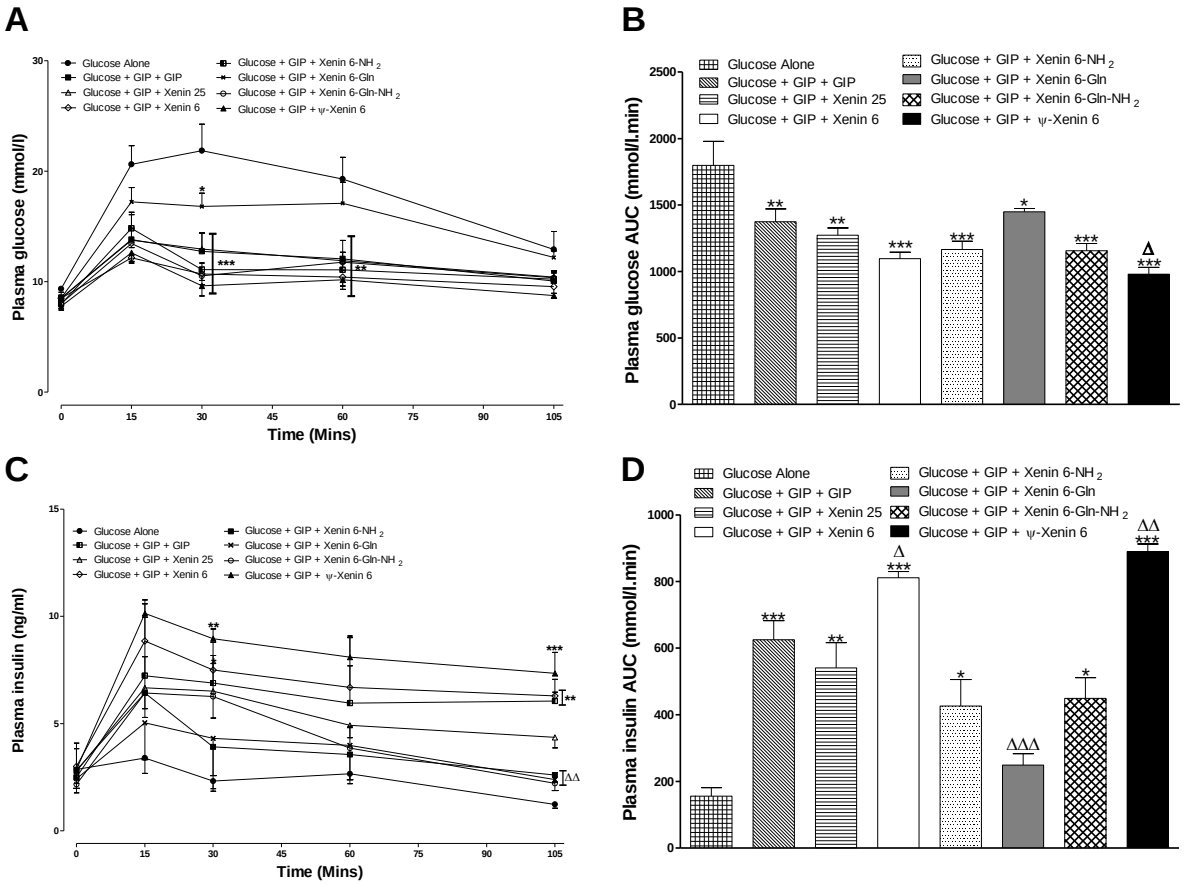
Cumulative food intake was measured in 18 h fasted mice at 30, 60, 90, 120, 150, and 180 min after i.p. injection of saline vehicle (0.9% w/v NaCl), xenin-25, xenin-6, xenin-6-NH_2, xenin-6-Gln, xenin-6-Gln-NH_2 or Ψ-xenin-6 in combination with exendin-4 (both at 25 nmol/kg bw). Values represent means ± SEM (n=8). ***P< 0.001 compared to respective saline control. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to xenin-6.

Fig. 4.11 Effects of xenin hexapeptides on glucose and insulin concentrations in lean mice.



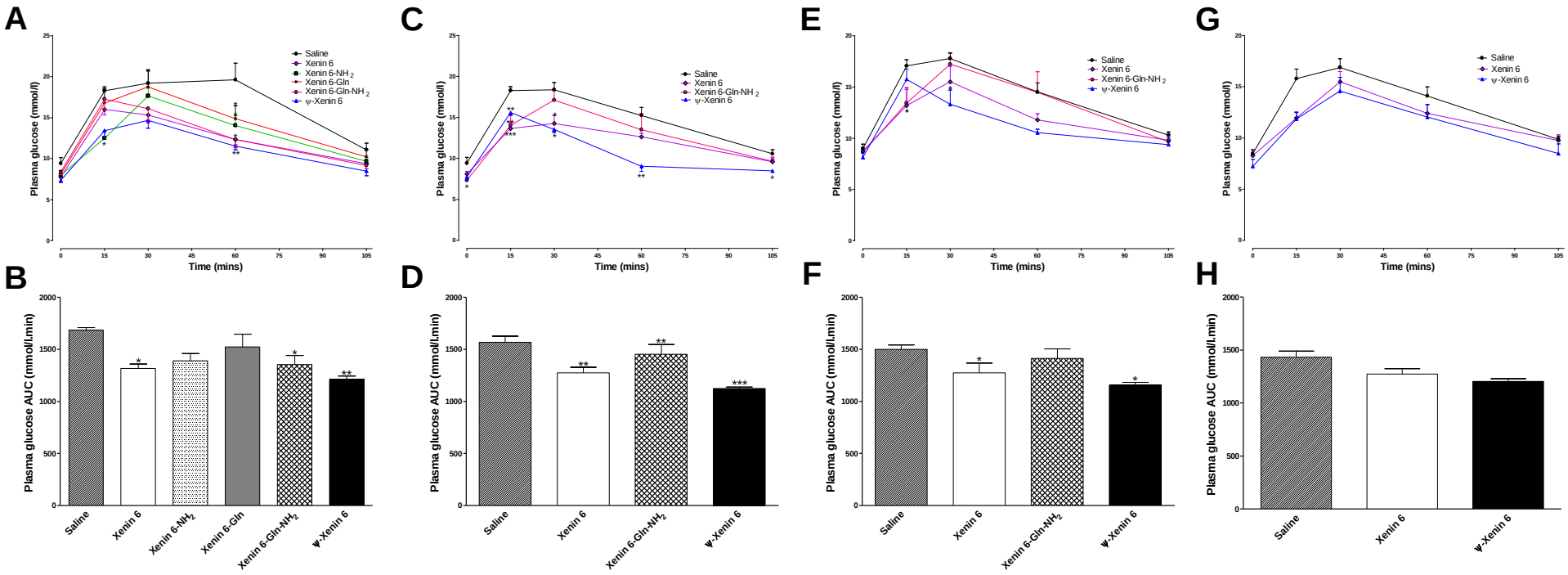
(A) Blood glucose and (C) plasma insulin concentrations were measured immediately before and 15, 30, 60 and 105 min after i.p. injection of glucose alone (18 mmol/kg bw) and test hexapeptides (each at 25 nmol/kg bw) in 4 h fasted mice. (B,D) Glucose and insulin AUC values for 0-105 min post injection. Values represent mean ± SEM for 6 mice. *P<0.05, **P<0.01 and ***P<0.001 compared to glucose alone. $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to xenin 25. $^{\Omega}$P<0.05 compared to xenin 6.

Fig. 4.12 Effects of xenin hexapeptides in combination with GIP on glucose and insulin concentrations in lean mice.



(A) Blood glucose and (C) plasma insulin concentrations were measured immediately before and 15, 30, 60 and 105 min after i.p. injection of glucose alone (18 mmol/kg bw) and combined injection of GIP and test peptides (each at 25 nmol/kg bw) in 4 h fasted mice. (B, D) Glucose and insulin AUC values for 0-105 min post injection. Values represent mean ± SEM for 6 mice. *P<0.05, **P<0.01 and ***P<0.001 compared to glucose alone. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to GIP control.

Fig. 4.13 Persistent glucose lowering effects of xenin hexapeptides in lean mice.



(A,C,E,G) Blood glucose concentrations were measured immediately before and 15, 30, 60 and 105 min following i.p. glucose load (18 mmol/kg bw) in 4 h fasted mice injected with saline vehicle or test peptides (each at 25 nmol/kg bw) 2 (A), 4 (C), 8 (E) or 12 h (G) previously. (B,D,F,H) Blood glucose AUC values for 0-105 min post injection. Values represent mean ± SEM for 6 mice. *P<0.05, **P<0.01 and ***P<0.001 compared to glucose alone.

Chapter 5

A pseudohexapeptide xenin analogue, Ψ-xenin-6, augments the antidiabetic benefits of the DPP-4 inhibitor sitagliptin in high fat fed mice

5.1 SUMMARY

The previous chapter characterised the biological properties of an enzymatically stable, C-terminal hexapeptide fragment of the gut hormone xenin, namely Ψ-xenin-6, including ability to potentiate the bioactivity of the incretin hormone GIP. Given that the primary therapeutic target of clinically approved DPP-4 inhibitors is augmentation of the incretin effect, the present study assessed the capacity of Ψ-xenin-6 to enhance the antidiabetic efficacy of sitagliptin in high fat fed (HFF) mice. Individual administration of either sitagliptin or Ψ-xenin-6 alone to HFF mice for 18 days resulted in numerous benefits including, reduced body weight gain ($P<0.05$), increased circulating and pancreatic insulin ($P<0.001$), improved glucose tolerance ($P<0.01$) and returning circulating glucose as well as pancreatic islet architecture similar to that observed in lean controls. As expected, sitagliptin therapy was associated with elevated circulating GIP and GLP-1 levels, with concurrent Ψ-xenin-6 not elevating these or enhancing DPP-4 inhibitory activity of the drug. However, combined sitagliptin and Ψ-xenin-6 therapy in HFF mice was associated with further notable benefits on metabolism, beyond that observed with either treatment alone. As such, combination therapy resulted in body weight change similar to lean controls, more pronounced and rapid benefits on circulating glucose and insulin as well as additional improvements in attenuating gluconeogenesis. Similarly, favourable effects on pancreatic islet architecture and peripheral insulin sensitivity were more apparent with combined therapy. Furthermore, expression of hepatic genes involved in gluconeogenesis and insulin action, such as *AKT1*, *FGF21*, *G6Pase*, *IRS-1* and *PEPCK* were partially, or fully restored to normal levels by treatment regimens, with beneficial effects being more prominent in combination treatment group. These data demonstrate for the first time that combined treatment with Ψ-xenin-6 and sitagliptin

offers clear antidiabetic advantages, which merit further consideration as a potential therapeutic option for T2DM.

5.2 INTRODUCTION

DPP-4 inhibitors are an orally available class of drugs, clinically approved for the treatment of T2DM [Scott, 2017]. The primary therapeutic benefit of DPP-4 inhibitors in T2DM relates to preventing degradation and subsequent loss of bioactivity, of the endogenous intestinal-derived incretin hormones, GLP-1 and GIP [Deacon, 2019]. Thus, GLP-1 and GIP stimulate glucose-dependent insulin secretion and improve glycaemic status, resulting in reduced glycated haemoglobin concentrations [Baggio and Drucker, 2007]. These hormones also exert a number of extrapancreatic actions that have beneficial glucose homeostatic effects [Baggio and Drucker, 2007; O'Harte *et al.* 1998].

However, a key pathophysiological aspect of T2DM relates to an impaired incretin effect [Holst and Ørskov, 2004]. As such, circulating levels of GLP-1 are believed to be reduced in T2DM, while the insulinotropic effects of GIP are severely diminished [Knop *et al*. 2007a]. DPP-4 inhibition will increase circulating concentrations of biologically active GLP-1 [Ahrén, 2007], but does not correct GIP insensitivity in T2DM. In this regard, a related 25 amino acid gut-derived hormone known as xenin, has been shown to augment the biological actions of GIP *in vitro* and *in vivo* [Wice *et al*. 2010; Martin *et al*. 2012; Gault *et al*. 2015a]. In addition, xenin also independently stimulates insulin secretion and supresses appetite [Taylor *et al.* 2010; Martin *et al.* 2012, 2014, 2016; Gault *et al*. 2015; Craig *et al.* 2018], which would be further advantageous in T2DM. Unlike GIP and GLP-1, xenin is not degraded by DPP-4

[Taylor *et al.* 2010], and thus DPP-4 inhibitors do not alter circulating xenin levels. However, enzymatic degradation fragment peptides of xenin are present in plasma [Martin *et al.* 2012], with some of these shown to retain the parent peptide biological action profile [Martin *et al.* 2014,2016; Parthsarathy *et al*. 2016; Craig *et al.* 2019]. In this regard, xenin-6 appears to be the shortest xenin form that recapitulates the key physiological actions of xenin at the level of the endocrine pancreas [Craig *et al.* 2019].

Recent studies in our laboratory [Craig *et al.* 2019; Chapter 4] have followed up on the initial characterisation of an enzymatically stabilised xenin-6 form, namely Ψ-xenin-6 [Feurle *et al.* 2003]. Ψ-xenin-6 is a C-terminal hexapeptide of xenin with a reduced pseudopeptide bond (CH_2NH) positioned between the first two N-terminal amino acid residues that possesses a significantly extended pharmacokinetic profile [Feurle *et al.* 2003]. Chapter 4 demonstrated that Ψ-xenin-6 stimulated insulin secretion from *in vitro* and *ex vivo* systems, promoted beta cell proliferation and survival, augmented glucose homeostasis, insulin secretion and satiety in rodents as well as amplifying the insulinotropic actions of GIP [Craig *et al.* 2019; Chapter 4]. Thus, combination therapy with a DPP-4 inhibitor and Ψ-xenin-6 represents an exciting potential therapeutic option for T2DM. Recent positive advances in the field of peptide hormone therapeutics and T2DM relate to the targeting of multiple receptor signalling pathways [Bhat *et al.* 2013; Irwin *et al*. 2015; Khajavi *et al.* 2017; Coskun *et al.* 2018; Frias *et al.* 2018]. Such strategies linked to observations of rapid remission of T2DM subsequent to certain types of bariatric surgeries, being directly associated with distinct changes in the secretion and action of numerous gut-derived peptide hormones [Vincent and Le Roux, 2008; Knop and Taylor, 2013].

In a distinct progression from previous work reported in this thesis (Chapter 4), in the present chapter a novel and targeted combinational therapeutic approach, employing Ψ-xenin-6 administration alongside the established DPP-4 inhibitor sitagliptin has been created. It is hypothesised that the complementary biological actions of both compounds would significantly improve diabetic status beyond that observed with either treatment alone. Thus, the major benefit of sitagliptin in diabetes therapy relates specifically to GLP-1 induced effects [Bennettt, 2018], whereas Ψ-xenin-6 will promote additional antidiabetic actions of the increased GIP levels encouraged by sitagliptin. As such, the primary objective was to examine therapeutic efficacy of 18-day combined administration of Ψ-xenin-6 and sitagliptin in HFF mice. Effects on body weight, energy intake, circulating glucose, insulin, GIP and GLP-1 as well as glucose and pyruvate tolerance, peripheral insulin sensitivity, expression of key genes involved in liver glucose metabolism and insulin action, as well as DPP-4 activity and pancreatic islet morphology were assessed.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Sub chronic studies were performed using male C57BL/6 mice (10-12 weeks old). All animals were maintained as described previously in Sections 2.13 and 2.13.3.

5.3.2 Experimental design

Prior to commencement of sub-chronic study, a combination of high fat diet and low dose STZ injections were used to develop a diet-induced type 2 diabetes mellitus animal model with increased loss of pancreatic beta cells, as described in Section 2.13.3. Figure 5.1 shows the experimental timeline for this study. Treatment

regimens were conducted as described in Section 2.16.1. Cumulative energy intake, body weight, non-fasting circulating glucose and insulin concentrations were assessed at regular intervals during the study. At the end of the treatment period, glucose tolerance (18 mmol/kg bw; i.p.; 18 h-fasted mice), insulin sensitivity (25 U/kg bovine insulin; i.p.; non-fasted mice) and pyruvate tolerance (2 g/kg sodium pyruvate; i.p.; 18 h-fasted mice) were assessed. HOMA-IR, fasting glucose (mmol/L) x fasting insulin (mU/L) / 22.5, was also calculated as a surrogate marker of insulin resistance (for more detail see sections 2.16.2.1, 2.16.2.3 and 2.16.2.4). Terminal analyses included extraction of pancreatic tissue for determination of pancreatic insulin content (sections 2.16.6 and 2.16.7). In addition, liver tissue was processed for hepatic gene expression by qPCR after total RNA extraction as described previously in sections 2.16.11 and 2.16.12). Real-time ready qPCR target-specific primers (Roche Diagnostics, West Sussex, UK) included: *PEPCK*, *G6pase*, *IRS-1*, *FGF21* and *AKT1*. β-actin was used as internal control for normalisation.

5.3.3 Biochemical analysis

Blood samples were collected as described (Section 2.15). Glucose and insulin were assayed as described in Sections 2.11.2 and 2.15. Total GLP-1 and GIP plasma concentrations were determined using specific ELISAs as described in Sections 2.16.8. DPP-4 activity of terminal plasma, as well as sitagliptin and Ψ-xenin-6 alone and in combination *in vitro* (both at 10^{-6} mol/l), was determined using a fluorometric assay that measures free 7-amino-4-methyl-coumarin (AMC) (Section 2.16.10).

5.3.4 Statistical analysis

Statistical analysis was completed using GraphPad PRISM (Section 2.18).

5.4 RESULTS

5.4.1 Effects of 18-day treatment of Ψ-xenin-6, sitagliptin or a combination of both compounds on body weight, fluid intake, energy intake, glucose and insulin concentrations in HFF mice

Increases of body weight were significantly ($P<0.01$ - $P<0.001$) reduced in HFF mice treated with Ψ-xenin-6, sitagliptin or a combination of both drugs by day 18, when compared to HFF saline controls (Figure 5.2A). Notably, in the combination treatment group, but not with monotherapy, percentage body weight increase was not significantly different from lean controls (Figure 5.2A). Water intake was significantly ($P<0.05$) reduced in all HFF treated groups on days 4, 18 and 18, when compared to HFF controls (Figure 5.2B). Energy intake was elevated ($P<0.01$ - $P<0.001$) in all HFF mice compared to lean controls, but not significantly different between HFF groups (Figure 5.2C). Non-fasting glucose levels were reduced to lean controls in a time-dependent manner by all treatments, but this was more prominent in combined treatment group with benefits evident from day 12 (Figure 5.3A). In addition, all HFF treatment groups exhibited progressive increases in insulin concentrations, with levels significantly ($P<0.05$ - $P<0.001$) elevated compared to HFF saline controls from day 7 onwards (Figure 5.3B). Moreover, in HFF mice treated with Ψ-xenin-6 in combination with sitagliptin, circulating insulin was not significantly different to lean control mice by day 18 (Figure 5.3B) and were significantly increased ($P<0.05$) compared to all other treatment groups (Figure 5.3B).

5.4.2 Effects of 18-day treatment of Ψ-xenin-6, sitagliptin or a combination of both compounds on glucose and pyruvate tolerance, insulin sensitivity as well as pancreatic insulin content in HFF mice

Treatment of HFF mice with Ψ-xenin-6, sitagliptin or a combination of both drugs for 18 days resulted in reductions ($P<0.05$ - $P<0.01$) of individual and overall glucose levels when compared to saline controls following i.p. glucose load (Figure 5.4A,B). However, glucose disposal was still impaired in all HFF mice when compared to lean controls (Figure 5.4A,B). Interestingly, corresponding glucose-induced insulin levels were not significantly different between all groups of mice at each of the observed time points (Figure 5.4C). However, overall AUC insulin concentrations were elevated ($P<0.01$) in saline treated HFF mice when compared to all other groups (Figure 5.4D). When challenged with a pyruvate load on day 18, only combined treatment with Ψ-xenin-6 and sitagliptin resulted in significant decrease ($P<0.05$ - $P<0.001$) in glucose levels when compared to HFF controls (Figure 5.5A). Overall blood glucose AUC values were reduced ($P<0.05$ - $P<0.001$) by all treatment interventions, but still elevated ($P<0.01$ - $P<0.001$) when compared to lean control mice (Figure 5.5B). However, combination treatment was significantly reduced ($P<0.05$) when compared to sitagliptin monotherapy (Figure 5.5B). Peripheral insulin sensitivity was improved ($P<0.05$ - $P<0.001$) by all treatment interventions (Figure 5.6A,B), although administration of sitagliptin alone, or in combination with Ψ-xenin-6, resulted in similar glucose-lowering effects of exogenous insulin as observed in lean mice (Figure 5.6A,B). HOMA-IR was also significantly ($P<0.05$ - $P<0.01$) improved in all HFF treatment groups, and not different from lean control mice (Figure 5.6C). Pancreatic insulin content was similar to lean control mice in all

HFF treatment groups, and significantly elevated (P<0.001) when compared to saline treated HFF mice (Figure 5.6D).

5.4.3 Effects of 18-day treatment of Ψ-xenin-6, sitagliptin or a combination of both compounds on pancreatic islet histology in HFF mice

Images of pancreatic islets stained for insulin and glucagon from all groups of mice are shown in Figure 5.7A-E. Visual inspection of these images illustrates marked reduced insulin staining in HFF saline treated mice when compared to lean controls (Figure 5.7A,B). Following appropriate quantification, it was revealed that HFF saline mice had significantly reduced overall islet (P<0.05) and beta cell (P<0.01) areas when compared to lean controls (Figure 5.8A,B). All treatment interventions returned islet and beta cell area to normal levels (Figure 5.8A,B). Alpha cell area was increased in HFF saline (P<0.001) and sitagliptin (P<0.05) treated mice, but similar to lean controls in HFF mice treated with Ψ-xenin-6 alone, or in combination with sitagliptin (Figure 5.8C).

Beta to alpha cell ratios were significantly (P<0.001) increased by all treatments when compared to HFF controls, and by Ψ-xenin-6 alone or in combination with sitagliptin, when compared to sitagliptin alone (P<0.001), but still reduced (P<0.001) when compared to lean control mice (Figure 5.8D). Islet size distribution was comparatively similar in all groups of mice, but all HFF mice administered treatment did have reduced (P<0.05 - P<0.01) numbers of smaller sized islets when compared to saline HFF controls (Figure 5.8E).

5.4.4 Effects of 18-day treatment of Ψ-xenin-6, sitagliptin or a combination of both compounds on expression of hepatic genes involved in gluconeogenesis and insulin action in HFF mice

As expected, high fat feeding increased ($P<0.01$ and $P<0.05$, respectively) hepatic expression of *PEPCK* and *G6pase* (Figure 5.9A,B). All treatment interventions significantly ($P<0.05$) reduced *pEpCK* mRNA levels (Figure 5.9A), but only combined Ψ-xenin-6 and sitagliptin therapy reduced ($P<0.05$) *G6pase* expression, and returned expression of both genes to lean control levels (Figure 5.9A,B). In addition, *IRS-1* hepatic expression levels were increased ($P<0.01$) in all HFF treatment groups when compared to saline controls (Figure 5.9C). Interestingly, only combination therapy increased ($P<0.001$) *FGF21*, and decreased ($P<0.01$) *AKT1*, hepatic mRNA levels compared to HFF control mice (Figure 5.9D,E). Indeed, expression levels of both genes were similar to lean control mice in the combined Ψ-xenin-6 and sitagliptin treatment group (Figure 5.9D,E).

5.4.5 Effects of 18-day treatment of Ψ-xenin-6, sitagliptin or a combination of both compounds on GLP-1 and GIP concentrations as well as DPP-4 activity in HFF mice

Following the 18-day treatment regimen, all sitagliptin treated mice had significantly ($P<0.01$ and $P<0.001$) increased circulating total GIP concentrations (Figure 5.10B). Interestingly, Ψ-xenin-6 therapy also increased ($P<0.01$) GIP levels, but did not affect GLP-1 concentrations (Figure 5.10A,B). However, the combination treatment group did not present with GIP levels greater than with those with sitagliptin therapy alone (Figure 5.10B). High fat feeding reduced ($P<0.001$) circulating GLP-1, but this was restored to normal lean control levels in all sitagliptin treated mice (Figure 5.10A).

Plasma DPP-4 activity was significantly ($P<0.05$) reduced in all HFF mice treated with sitagliptin when compared to HFF controls (Figure 5.10C). DPP-4 activity in Ψ-xenin-6 treatment group was not significantly altered when compared to lean or high fat control mice (Figure 5.10C). To confirm *in vivo* observations, DPP-4 activity of sitagliptin, Ψ-xenin-6 and a combination of both compounds was evaluated in the *in vitro* setting (Figure 5.10D). In harmony with data from our mouse study, Ψ-xenin-6 (10^{-6} mol/l) had no effect on DPP-4 activity and did not augment sitagliptin-induced reductions of DPP-4 activity (Figure 5.10D).

5.5 DISCUSSION

Although DPP-4 inhibitors have undoubted therapeutic benefits in T2DM [Elrishi et al. 2007], their overall efficacy is perhaps less striking than other clinically approved drugs [Rosenstock et al. 2010]. Taking into consideration that the primary pharmacological benefit of DPP-4 inhibition is enhancement of the incretin effect [Gallwitz, 2013], known to be severely perturbed in T2DM [Nauck et al. 1993], this is perhaps not overly surprising. In the current chapter, we sought to address this problem through concurrent administration of the DPP-4 inhibitor, sitagliptin, with the enzymatically stable xenin form, Ψ-xenin-6 [Feurle et al. 2003]. Ψ-xenin-6 represents a compound known to correct the underlying incretin defect in T2DM, specifically GIP insensitivity [Craig et al. 2019], as outlined in chapter 4.

As would be expected, sitagliptin and Ψ-xenin-6 therapy induced a number of benefits on metabolism in HFF mice. This is interesting, given that Ψ-xenin-6 was administered at a reduced molar concentration when compared to sitagliptin, and because HFF mice were also administered two low doses of STZ prior to treatment

intervention. Such benefits included reduced glucose levels as well as improved glucose tolerance and insulin sensitivity, with notable improvements in pancreatic islet architecture. Indeed, assessment of peripheral insulin sensitivity would have been even more positive, but for normal adaptive responses in these mice to prevent glucose concentrations falling towards life threatening hypoglycaemia levels, following exogenous insulin injection. As expected, positive effects of sitagliptin were linked to elevated circulating GIP and GLP-1 concentrations [Deacon *et al*. 1998]. Interestingly, Ψ-xenin-6 also increased GIP levels, which may be somewhat unexpected. As such, the secretory dynamics of intestinal K-cells are well established [Gribble and Reimann, 2019], although whether xenin exerts a positive autocrine effect on GIP secretion from K-cells is currently unknown. More importantly, elevated GIP and GLP-1 concentrations of the combined treatment group were not greater than with sitagliptin alone, confirming that benefits of combination therapy are not linked to increased incretin hormone levels.

Despite administration of the specific beta cell toxin STZ, each of the treatments, namely sitagliptin, Ψ-xenin-6 or a combination of both compounds, returned overall islet and beta cell areas, as well as pancreatic insulin content, to normal levels. It is intriguing to note that in a related HFF mouse model with STZ-induced defects of insulin secretion, similar benefits of pancreatic islet architecture were noted following sustained administration of an analogue of sitagliptin, namely des-fluoro-sitagliptin [Mu *et al.* 2006]. Notably, in this study by Mu and co-workers, des-fluoro-sitagliptin was administered for 11 weeks in the diet, as opposed to 18 days oral gavage in the current study. Thus, the oral gavage route ensures consistent dosing, drug delivery and faster onset of action, which may not always be the case with drug-

supplemented animal diets. Whether augmented pancreatic insulin content in the current study was due to increases in beta-cell proliferation, reductions of beta cell apoptosis or increased insulin production within beta cells still needs to be determined. However, the beneficial impact of such pancreatic changes was clearly apparent on circulating insulin concentrations, with steady augmentations being evident in the combined treatment group. In keeping with this, GIP, GLP-1 and xenin have been shown to independently stimulate insulin secretion [Schmidt *et al.* 1985; Taylor *et al.* 2010], as well as promote pancreatic beta cell growth and survival [Vilsbøll and Holst, 2004; Drucker, 2006; Khan et al. 2017a]. In addition to this, intra-islet synthesis and secretion of GLP-1 has previously been shown to play a fundamental role in appropriate pancreatic islet adaptations to various diabetes-like disease states [Vasu et al. 2014b; Moffett et al. 2014]. As such, it would have been interesting to assess pancreatic islet levels of both GLP-1 and GIP in the current study. Nonetheless, dual treatment with sitagliptin and Ψ-xenin-6 evoked rapid improvements in glycaemic status, beyond that observed with either treatment alone. Such benefits were more apparent in the fed, as opposed to fasted state, as evidenced during glucose tolerance testing that revealed no differences between the treatment regimens. This could simply be a consequence of the primary metabolic benefits of GIP, GLP-1 and xenin being more associated with the postprandial setting [Chowdhury *et al.* 2013; Irwin and Flatt, 2015].

Although benefits on circulating insulin and pancreatic morphology were evident, none of the treatments increased the insulin response to exogenous glucose on day 18. Furthermore, there was a significant improvement in peripheral insulin sensitivity in all treated HFF mice, suggesting that improvements in glycaemic control were not

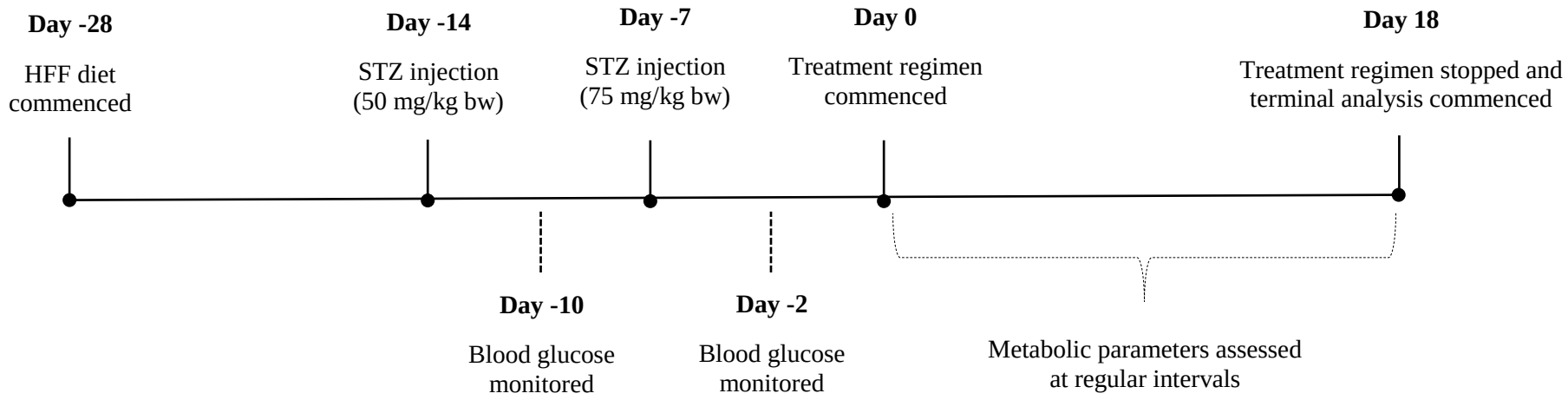
solely a result of pancreatic-related benefits, and could be related to the weight loss induced by each treatment. Indeed, hepatic expression of *IRS*-1 was augmented to a similar level by all treatment interventions. Moreover, combined sitagliptin and Ψ-xenin-6 therapy also increased *FGF21* expression in the liver and returned mRNA levels of hepatic *AKT1* to that of lean controls, both of which would improve insulin action [Kim *et al.* 2000; Emanuelli *et al.* 2014]. However, whether these positive changes in gene expression are independent of body weight loss still needs to be determined. In keeping with insulin action, hepatic gluconeogenesis was decreased in all treatment groups, but particularly by combined therapy, as established through assessment of pyruvate tolerance. In this respect, hyperinsulinaemic clamp studies may have given a more consistent readout of hepatic glucose production, but are beyond the scope of the current study. Nonetheless, irregular carbohydrate metabolism and inappropriate pyruvate flux are well known to be associated with diabetes and obesity [Gao *et al.* 2009; Singla *et al.* 2010]. To further support the notion that sitagliptin and Ψ-xenin-6 diminish hepatic gluconeogenesis, we determined expression of *PEPCK* and *G6pase* in liver tissue, key signalling molecules in the gluconeogenesis pathway [Lochhead *et al.* 2000]. As such, *PEPCK* and *G6Pase* are fundamental in the initial and final steps, respectively, of the gluconeogenic pathway [Hers and Hue, 1983]. Only combined sitagliptin and Ψ-xenin-6 treatment significantly reduced expression of both genes, and although some obvious restraint is required in interpretation especially since individual treatments also evoked reductions in the expression of these genes, this does suggest that the expression and subsequent biological function of these proteins could be a major factor in the improvement of gluconeogenesis. In addition, it would also have been interesting to assess plasma and tissue lipid status in these mice, as well as genes involved in lipid

flux. Moreover, assessment of related protein expression would be required to confirm overall relevance of the observed changes in gene expression.

Despite knowledge of the weight neutral effects of DPP-4 inhibitors [Foley and Jordan, 2010], as well as lack of effect of prolonged administration of enzymatically stable versions of xenin on body weight in diabetic mice [Gault *et al.* 2015a; Martin *et al.* 2016; Parthsarathy *et al.* 2016], both sitagliptin and Ψ-xenin-6 reduced body weight gain in the current setting. Moreover, combined treatment in HFF mice resulted in body weight change that was not different to lean controls, despite similar energy intake in all groups of HFF mice. In keeping with this, lack of effect of sitagliptin and xenin peptides on feeding has previously been observed in rodents using the same dosing regimens employed here [Taylor *et al*. 2010*;* Gault *et al.* 2015b; Hasib *et al.* 2017]. In view of the role of obesity in the development of T2DM [Al-Goblan *et al.* 2014], and the comparable increasing prevalence of both conditions [Agha and Agha, 2017], this more pronounced body weight lowering effect could represent an attractive therapeutic effect of combined therapy. As such, it is well recognised that the central pathways controlling energy balance display notable plasticity [Irwin and Flatt, 2015], thus concurrent positive modulation of signal transduction proteins linked to both compounds may be essential to yield such appreciable benefits. A reduction of body weight could also be a contributing factor to improved metabolic effects of the combination treatment [Wilding, 2014], although further assessment of body composition, locomotor activity and metabolic rate, including energy expenditure, would be required to fully assess this.

Taken together, data presented within the current study supports the view that the antidiabetic benefits of DPP-4 inhibitors can be augmented. Importantly, assessment of DPP-4 activity on day 18, together with *in vitro* observations, demonstrated that Ψ-xenin-6 does not augment DPP-4 inhibitory activity of sitagliptin. Given that incretin hormone levels were also not increased by dual administration of Ψ-xenin-6 and sitagliptin, it suggests that the likely benefit of combined therapy is linked to the augmentation of GIP bioactivity by Ψ-xenin-6, as has been previously established [Craig *et al*. 2019]. Observations of improved effects of combined Ψ-xenin-6 and sitagliptin treatment on body weight, circulating glucose and insulin, attenuation of gluconeogenesis, as well as improvements in pancreatic islet morphology are encouraging, especially over a relatively short treatment period of 18 days. Clinical studies already confirm some enhanced antidiabetic efficacy when using combinational therapies that incorporate DPP-4 inhibitor drugs [Goldstein *et al.* 2007; Barnett *et al.* 2015], in line with NICE treatment guidelines, corroborating proof-of-concept. Taking into account that Ψ-xenin-6 positively regulates the primary therapeutic target of DPP-4 inhibitors, unlike other available antidiabetic medications; the current therapeutic regimen represents an attractive novel option for T2DM that warrants further investigation.

Figure 5.1 Timeline for the experimental study



Streptozotocin (STZ) treatment: Two low dose STZ injections (50 mg/kg bw & 75 mg/kg bw, i.p, in sodium citrate buffer, pH 4.5) at one week intervals

Treatments started on Day 0 (n=8);

Group 1 (Lean control) – Lean control mice on normal diet received saline vehicle (0.9% w/v NaCl, i.p.) for 18 days

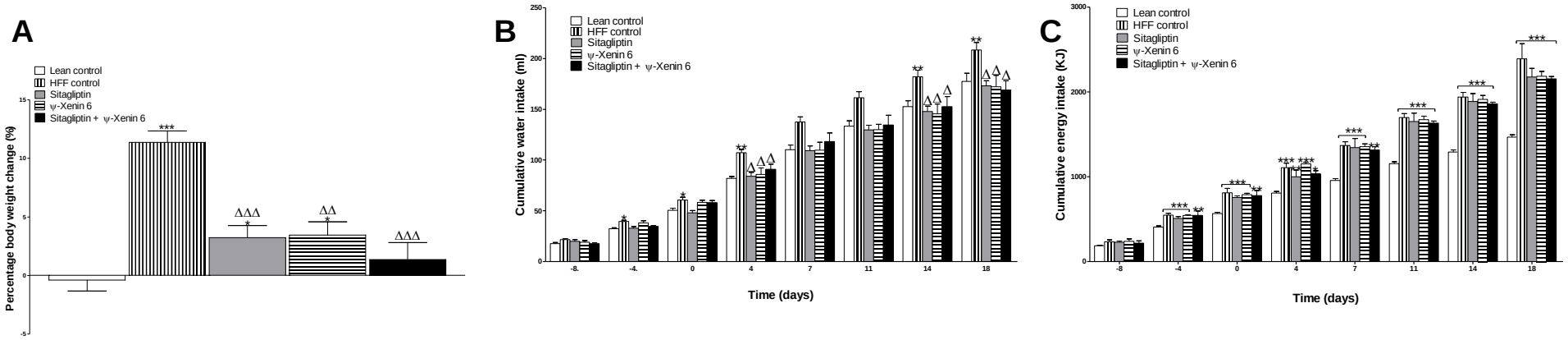
Group 2 (HFF control) – HFF saline vehicle (0.9% w/v NaCl, i.p.) for 18 days

Group 3 (Sitagliptin) – HFF Sitagliptin (50 mg/kg bw, p.o, once daily) for 18 days

Group 4 (Ψ-xenin-6) – HFF Ψ-xenin-6 (25 nmol/kg bw, i.p., twice daily) for 18 days

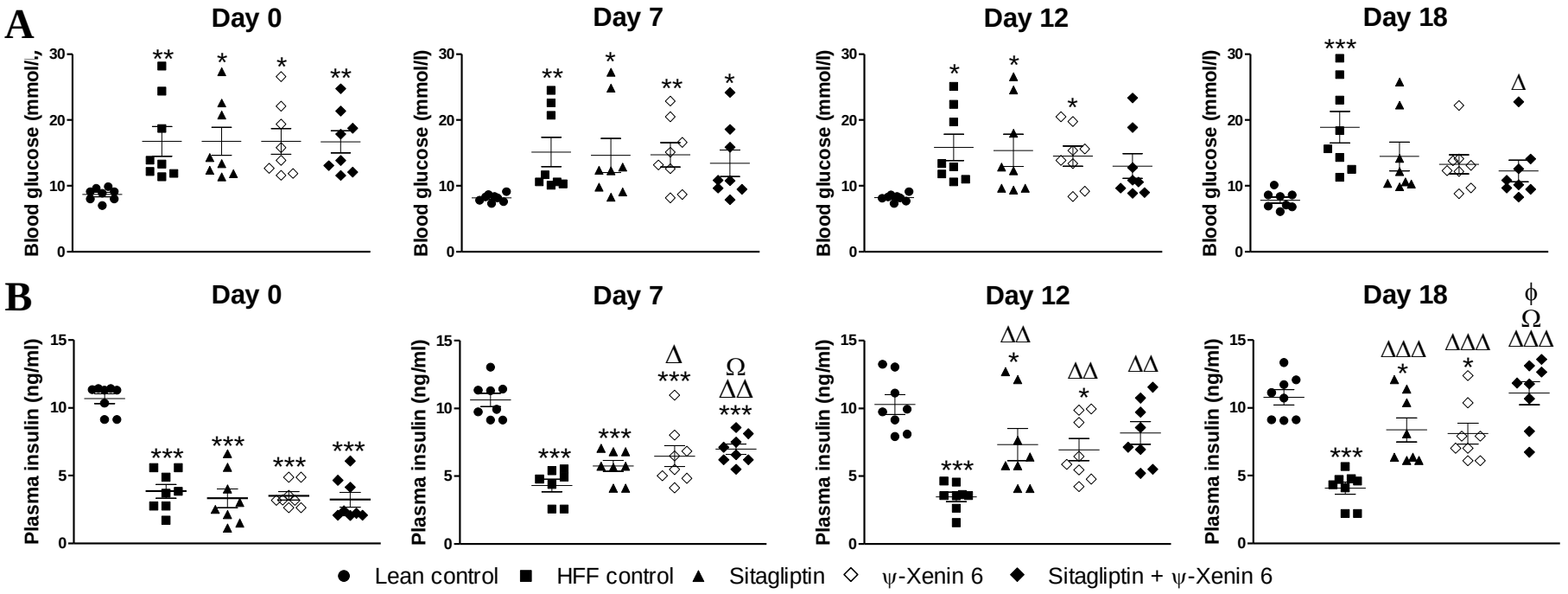
Group 5 (Sitagliptin + Ψ-xenin-6) – HFF Sitagliptin + Ψ-xenin-6 (50 mg/kg bw, p.o and 25 nmol/kg bw, i.p. respectively) for 18 days

Figure 5.2 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or a combination of both compounds on (A) body weight, (B) water intake and (C) energy intake in HFF mice



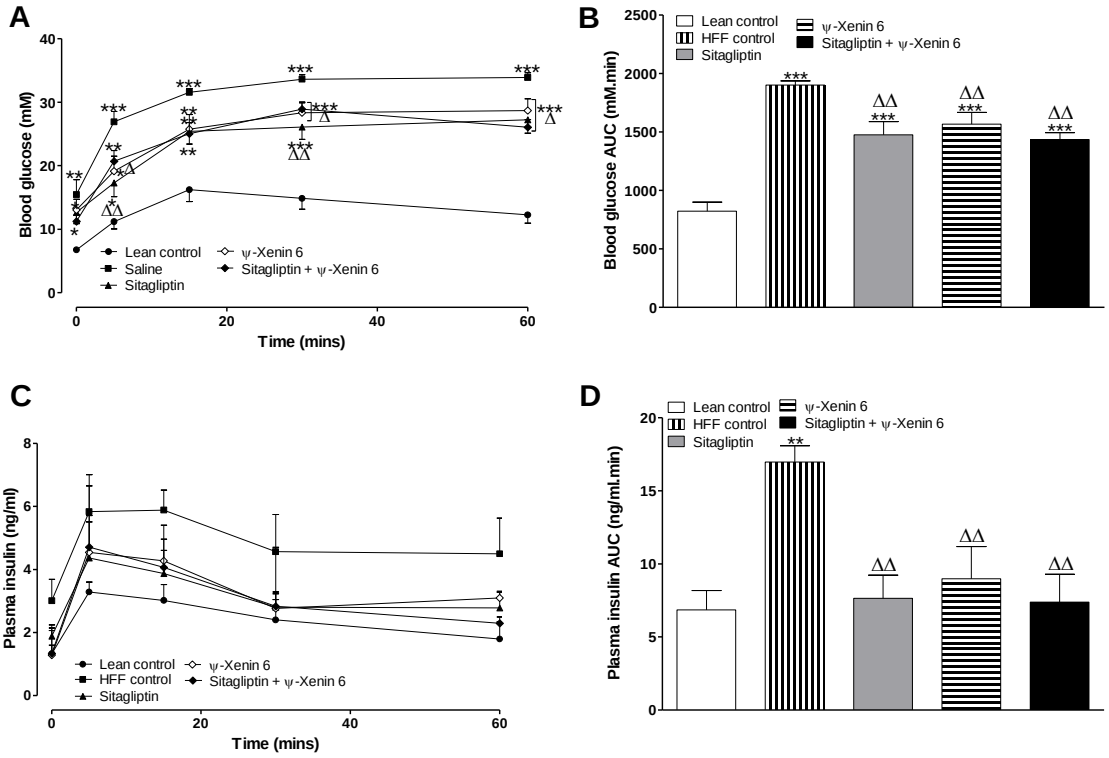
Parameters were measured during 18-days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p., twice daily) or a combination of both compounds using the same dosing regimens. Values are mean ± SEM for eight mice. *P<0.05, **P<0.01 and ***P<0.001 compared to lean controls. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to HFF saline controls.

Figure 5.3 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or a combination of both compounds on (A) glucose and (B) insulin concentrations in HFF mice.



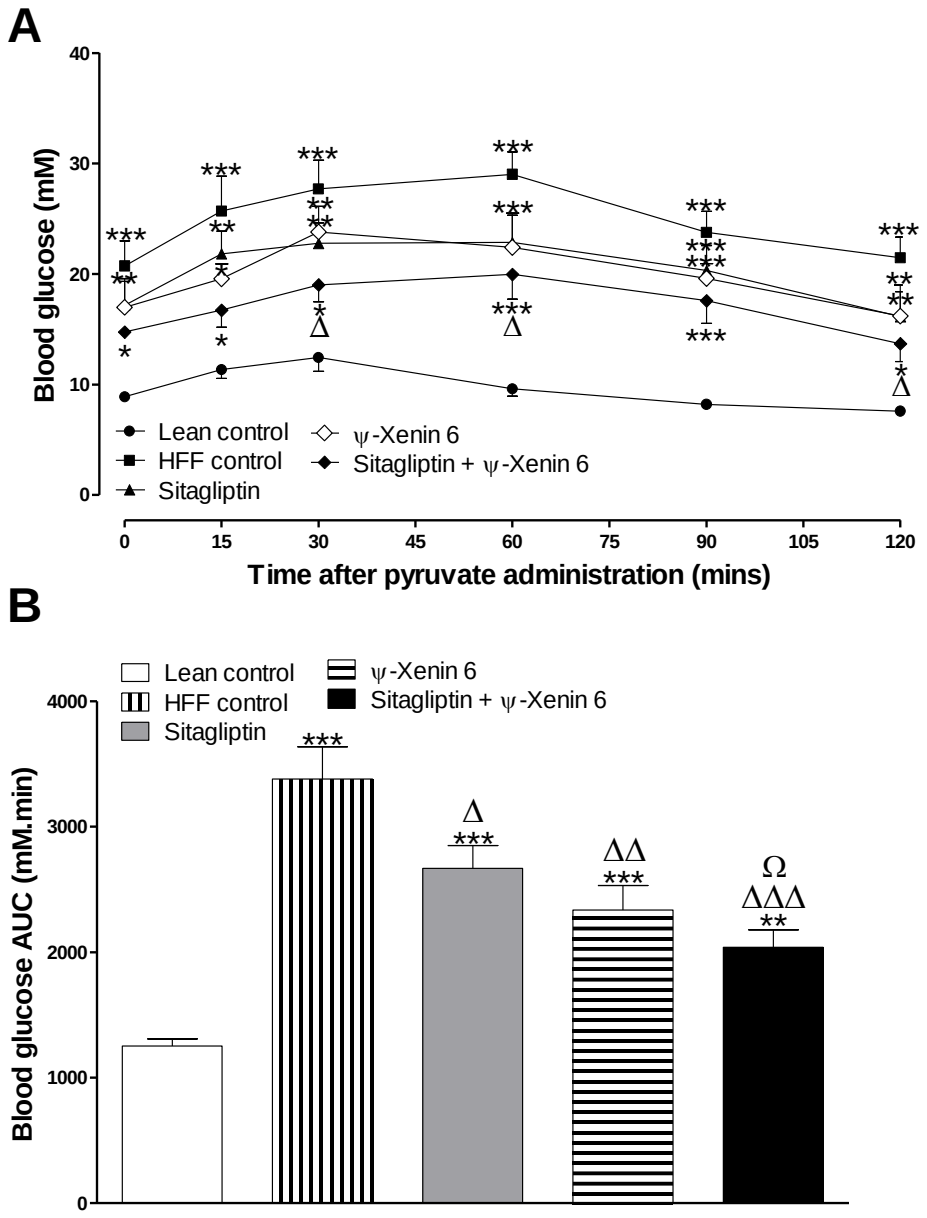
Parameters were measured during 18-day treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p. twice daily) or a combination of both compounds using the same dosing regimens. Values are mean ± SEM for eight mice. *P<0.05, **P<0.01 and ***P<0.001 compared to lean controls. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to HFF saline controls. $^{\Omega}$P<0.05 compared to sitagliptin alone. $^{\phi}$P<0.05 compared to Ψ-xenin-6 alone.

Figure 5.4 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or a combination of both compounds on glucose tolerance and insulin secretion in HFF mice.



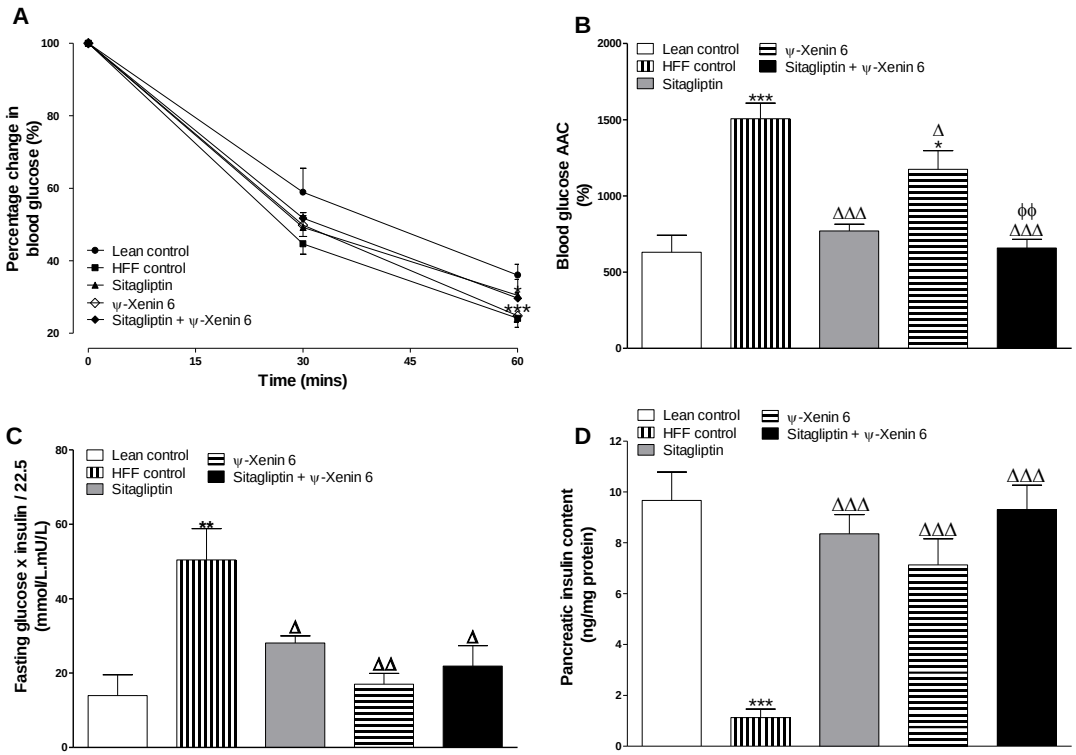
Parameters were assessed following 18 days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p. twice daily) or a combination of both compounds using the same dosing regimens. Glucose (18 mmol/kg bw) was administered by i.p. injection at t=0 min in 18 hour fasted mice. Glucose and insulin AUC values for 0–60 min post injection are also shown. Values are mean ± SEM for eight mice. *P<0.05, **P<0.01 and ***P<0.001 compared to lean controls. $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 compared to HFF saline controls.

Figure 5.5 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or combination of both compounds on pyruvate tolerance in HFF mice.



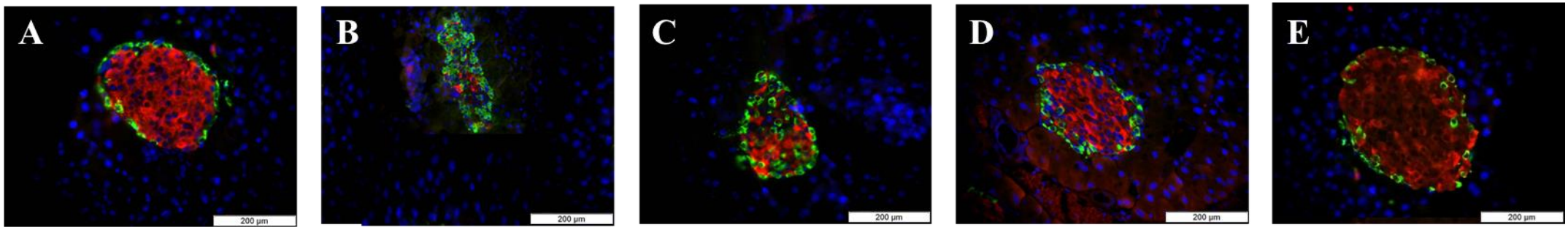
Parameters were assessed following 18-days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p. twice daily) or a combination of both compounds using the same dosing regimens. (A) Pyruvate (2 g/kg bw sodium pyruvate) was administered by i.p. injection at t=0 min in 18 hour fasted mice. (B) Blood glucose AUC values for 0–120 min post injection are shown. Values are mean ± SEM for eight mice. *P<0.05, **P<0.01 and ***P<0.001 compared to lean controls. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to HFF saline controls. $^{\Omega}$P<0.05 compared to sitagliptin alone.

Figure 5.6 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or a combination of both compounds on insulin sensitivity and pancreatic insulin content in HFF mice.



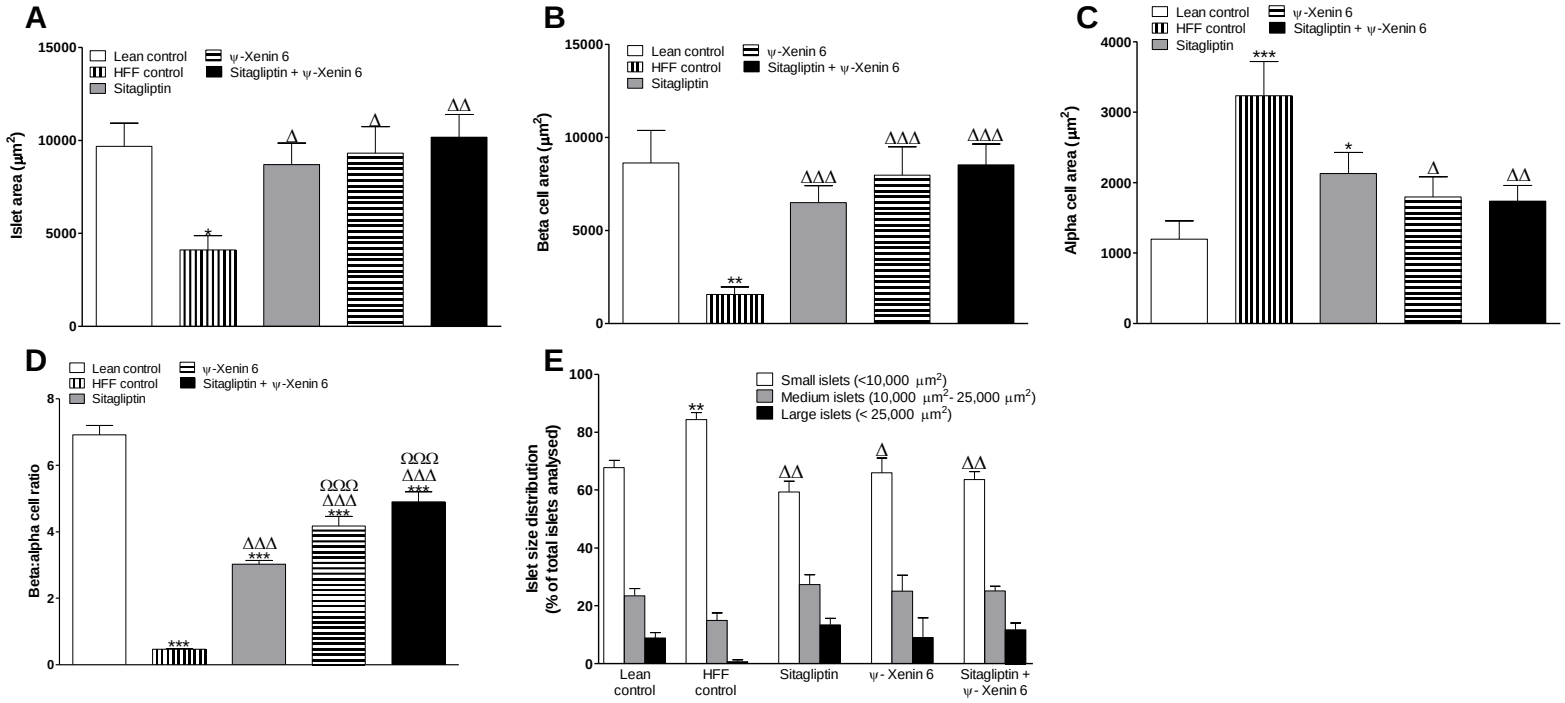
Parameters were assessed following 18-days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p. twice daily) or a combination of both compounds using the same dosing regimens. (A) Insulin (25 U/kg bw) was administered by i.p. injection at t=0 min in non-fasted mice. (B) Blood glucose AAC values for 0–60 min post injection. (C) HOMA-IR was calculated by the following equation: fasting glucose (mmol/L) x fasting insulin (mU/L) / 22.5. (D) Pancreatic insulin content was measured by RIA following acid-ethanol extraction. Values are mean ± SEM for eight mice. *P<0.05 and ***P<0.001 compared to lean saline controls. $^{\Delta}$P<0.05 and $^{\Delta\Delta\Delta}$P<0.001 compared to HFF saline controls. $^{\phi\phi}$P<0.01 compared to Ψ-xenin-6 alone.

Figure 5.7 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or a combination of both compounds on pancreatic islet architecture in HFF mice.



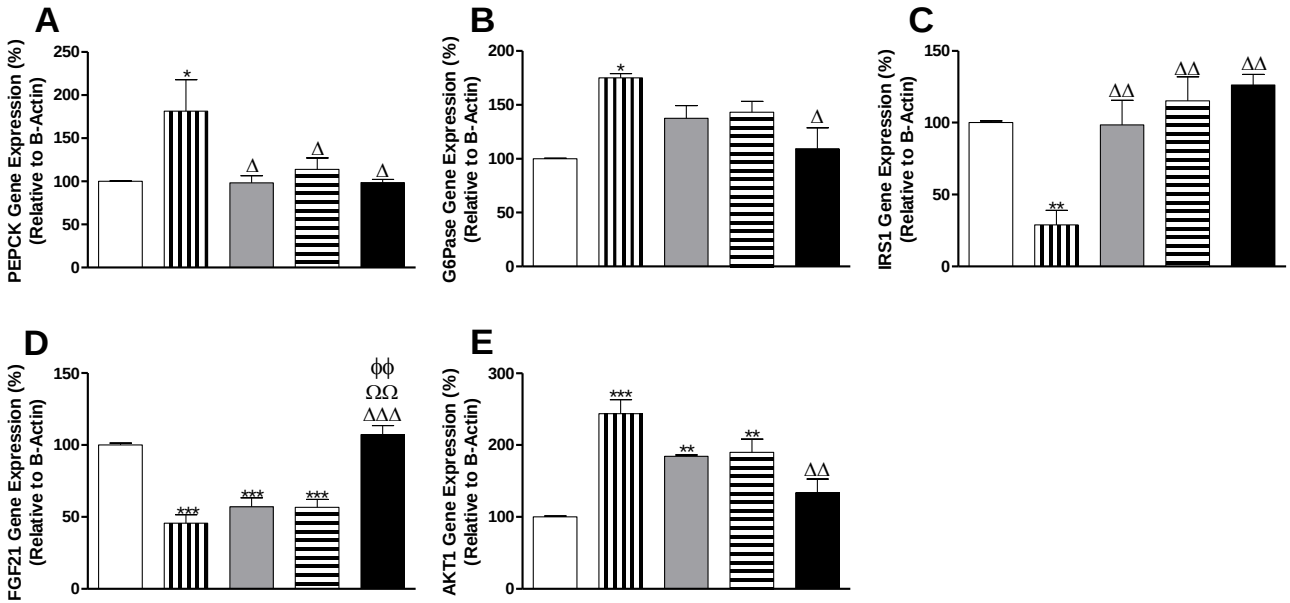
Images (20X) were captured following 18-days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p., twice daily) or a combination of both compounds using the same dosing regimens. Pancreatic islet images show insulin (red) and glucagon (green) immunoreactivity from (A) lean (B) high fat saline control, (C) sitagliptin, (D) Ψ-xenin-6, (D) combined treated high fat fed mice.

Figure 5.8 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or a combination of both compounds on pancreatic islet composition in HFF mice.



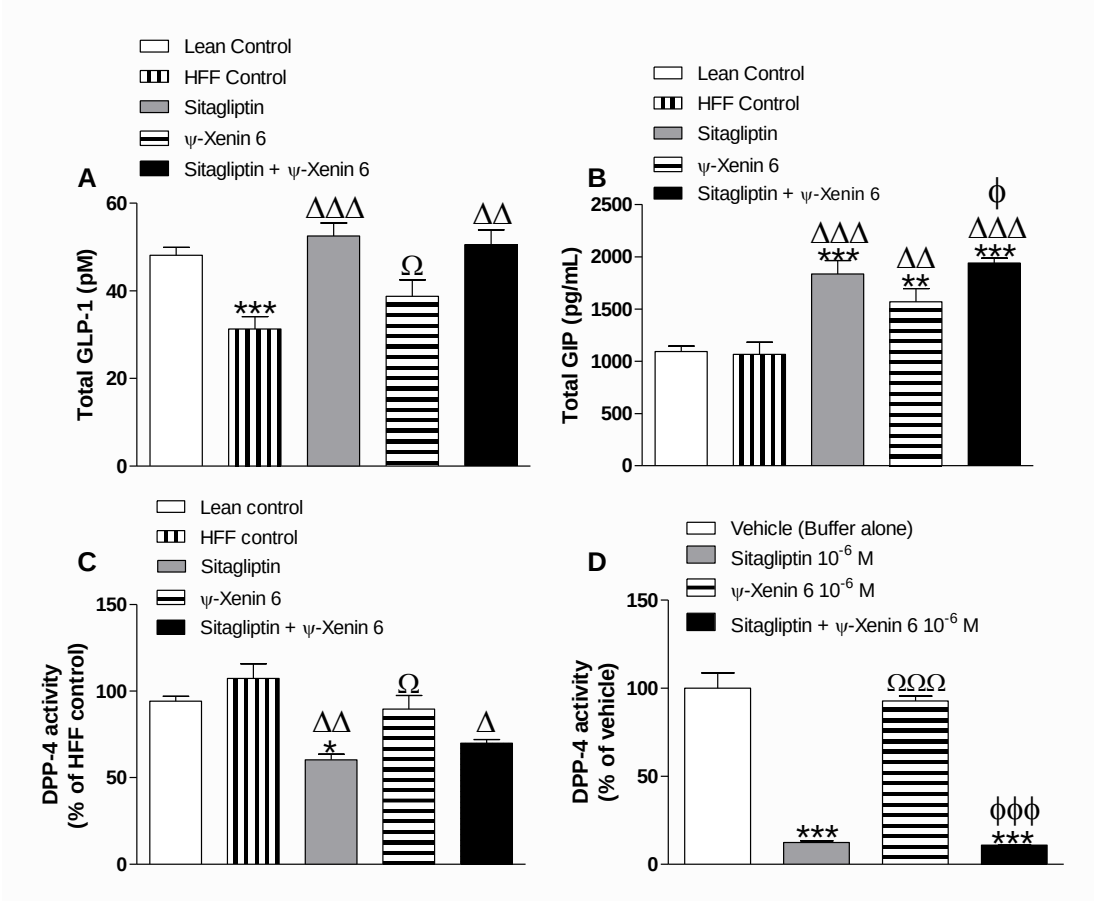
Parameters were assessed following 18-days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p. twice daily) or a combination of both compounds using the same dosing regimens. (A) Islet, (B) beta- and (C) alpha-cell areas, as well as (D) beta:alpha area cell area ratio and (E) islet size distribution were measured using Cell^F image analysis software. Values are mean ± SEM for eight mice. *P<0.05, **P<0.01 and ***P<0.001 compared to lean controls. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to HFF saline controls. $^{\Omega\Omega\Omega}$P<0.001 compared to sitagliptin alone.

Figure 5.9 Effects of 18-day treatment with Ψ-xenin-6, sitagliptin or a combination of both compounds on hepatic gene expression in HFF mice.



Expression was assessed following 18-days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p., twice daily) or a combination of both compounds using the same dosing regimens. mRNA expression of (A) phosphoenolpyruvate carboxykinase (*PEPCK*), (B) glucose 6-phosphatase (*G6pase*), (C) insulin receptor substrate-1 (*IRS-1*), (D) fibroblast growth factor 21 (*FGF21*) and (E) serine-threonine protein kinase (*AKT1*) were determined relative to control gene expression (β-actin). Values are mean ± SEM for eight mice. *P<0.05, **P<0.01 and ***P<0.001 compared with lean controls. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to HFF saline controls. $^{\Omega\Omega}$P<0.01 compared to sitagliptin alone. $^{\phi\phi}$P<0.01 compared to Ψ-xenin-6 alone.

Figure. 5.10 Effects of Ψ-xenin-6, sitagliptin or a combination of both compounds on total GLP-1 and GIP concentrations as well as DPP-4 activity in HFF mice.



(A-C) Parameters were assessed following 18 days treatment with sitagliptin (50 mg/kg bw, p.o., once daily), Ψ-xenin-6 (25 nmol/kg bw. i.p., twice daily) or a combination of both compounds using the same dosing regimens. Total plasma (A) GLP-1 and (B) GIP concentrations in plasma were determined using commercially available ELISA kits. (C) DPP-4 activity was measured in plasma using established procedures (Lindsay et al. 2005). (D) DPP-4 activity of all test compounds (10^{-6} mol/l) was also determined in the *in vitro* setting. Values are mean ± SEM for (A-C) eight mice or (D) four experimental replicates. *P<0.05, **P<0.01 and ***P<0.001 compared with lean or vehicle controls, as appropriate. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to HFF saline controls. $^{\Omega}$P<0.05 and $^{\Omega\Omega\Omega}$P<0.001 compared to sitagliptin alone. $^{\phi}$P<0.05 and $^{\phi\phi\phi}$P<0.001 compared to Ψ-xenin-6 alone.

Chapter 6

Combined modulation of TNP-470 with sitagliptin results in rapid restoration of normoglycaemia and enhances circulating GIP concentrations in diabetic mice.

6.1 SUMMARY

The therapeutic mechanism of methionine aminopeptidase 2 (MetAP2) inhibitors for obesity-diabetes has not yet been fully defined. Xenin, a K-cell derived peptide hormone, possesses an N-terminal Met amino acid residue and sub-chronic parenteral administration of long-acting xenin analogues improves obesity-diabetes. Thus, elevated xenin levels could represent a potential pharmacological mechanism of MetAP2 inhibitors. The present study examined the ability of the MetAP2 inhibitor, TNP-470, to augment the antidiabetic utility of the DDP-4 inhibitor, sitagliptin, in high fat fed (HFF) mice. TNP-470 (1 mg/kg) and sitagliptin (25 mg/kg) were administered once-daily alone, or in combination, to HFF mice for 18 days. Individual therapy with TNP-470 or sitagliptin resulted in numerous metabolic benefits including reduced ($P<0.05$ - $P<0.001$) glucose, increased ($P<0.01$ - $P<0.001$) circulating and pancreatic insulin and improved ($P<0.05$ - $P<0.001$) glucose tolerance, insulin sensitivity, pyruvate tolerance and pancreatic islet architecture. Further assessment of metabolic rate revealed that all treatments reduced ($P<0.01$) RER and increased ($P<0.05$ - $P<0.01$) locomotor activity, with energy expenditure increased ($P<0.05$ - $P<0.01$) in all mice receiving sitagliptin therapy. In addition, treatment with TNP-470 alone, or in combination with sitagliptin, reduced ($P<0.05$ - $P<0.01$) food intake and body weight, as well as elevating ($P<0.01$) plasma and intestinal xenin. Importantly, combined sitagliptin and TNP-470 therapy was associated with further significant ($P<0.05$) benefits beyond that observed by either treatment alone. This included more rapid restoration of normoglycaemia, superior glucose tolerance, increased circulating GIP concentrations and enhanced beta:alpha cell ratio. These data demonstrate that TNP-470 increases plasma and intestinal xenin levels, and augments the antidiabetic properties of sitagliptin.

6.2 INTRODUCTION

Chapter 5 demonstrated that the therapeutic efficacy of sitagliptin can be augmented through combination therapy with ψ-xenin-6. Sitagliptin, the first clinically approved DPP-4 inhibitor, is a highly selective and potent, orally available drug for the management of T2DM [Scott, 2017]. The primary pharmacological mechanism of sitagliptin concerns blocking DPP-4 mediated degradation, and subsequent loss of bioactivity, of the two incretin hormones, namely GLP-1 and GIP [Drucker, 2007b]. As such, a reduced postprandial insulinotropic incretin effect is a recognised significant physiopathological aspect of T2DM [Holst *et al*. 2011b]. Suggestions for this diminished incretin effect include reduced circulating GLP-1 levels, as well as blunted insulinotropic effects of GIP [Knop *et al*. 2007b]. DPP-4 inhibitors increase circulating levels of GLP-1 [Omar and Ahrén, 2014], with subsequent stimulation of insulin secretion and inhibition of glucagon secretion, as well as enhancing glucose utilisation and reducing hepatic glucose production [Omar and Ahrén, 2014]. However, this class of drug fails to correct the insensitivity of GIP in T2DM. Thus, it would be favourable if the biological actions of GIP were potentiated in DPP-4 related therapies, especially since recent observations reveal significant complimentary beneficial antidiabetic actions of these two hormones [Portron *et al*. 2017; Frias *et al*. 2018; Bastin and Andreelli, 2019].

In this respect, xenin, a peptide hormone secreted postprandially from the same intestinal K-cells as GIP, has been shown to augment GIP-mediated insulinotropic responses [Wice *et al*. 2010; Taylor *et al*. 2010; Martin *et al*. 2012, 2014; Parthsarathy *et al*. 2016; Craig *et al*. 2019]. As with GIP and GLP-1, a major problem with clinical exploitation of xenin relates to its short biological half-life due to enzymatic

degradation [Martin *et al*. 2014]. However, there is a relative dearth of information regarding the enzyme(s) responsible for degradation of xenin [Taylor *et al*. 2010; Martin *et al*. 2014]. With this in mind, methionine aminopeptidase 2 (MetAP2) is a cytosolic metalloenzyme that catalyses hydrolytic removal of N-terminal methionine (Met) residues from nascent proteins [Varland *et al*. 2015]. As such, xenin is a peptide hormone that possess an N-terminal Met amino acid residue [Martin *et al*. 2016]. More interestingly, orally available MetAP2 inhibitors have already been generated, such as TNP-470 [Kusaka *et al*. 1991; Kruger and Figg, 2000], with demonstrated therapeutic efficacy for obesity and T2DM [Sin *et al*. 1997; Bråkenhielm *et al*. 2004; White *et al*. 2012; Joharapurkar *et al*. 2014]. Intriguingly, this accords well with the established metabolic advantages of sustained xenin administration in animal models of T2DM [Gault *et al*. 2015a; Martin *et al*. 2016; Craig *et al*. 2019], implying that benefits of MetAP2 inhibition in diabetes could partly be due to enhanced xenin bioactivity. As such, combinational therapy with a DPP-4 inhibitor, such as sitagliptin, and a MetAP2 inhibitor like TNP-470, could represent a highly effective therapeutic strategy for T2DM. DPP-4 inhibition will augment circulating GIP and GLP-1 levels, whilst MetAP2 inhibition should help to re-establish the biological actions of GIP through increasing circulating xenin concentrations. Although peptidic, long-acting, enzymatically resistant forms of GIP, GLP-1 and xenin have been characterised [Gupta, 2013; Tatarkiewicz *et al*. 2014; Gault *et al*. 2015a; Craig *et al*. 2019], these require parenteral administration, whereas enzyme inhibitors are orally available which should enhance patient compliance.

Therefore, in the current study a novel treatment paradigm, through co-administration of TNP-470 with sitagliptin in HFF mice was created. We

hypothesised that the biological efficacy of combined administration should outweigh the benefits of either treatment alone. Effects on body weight, food and water intake, circulating glucose and insulin, glucose and pyruvate tolerance, insulin sensitivity were investigated following 18-days treatment in HFF mice. Impact of the treatment regimens on plasma GIP, GLP-1 and xenin as well as intestinal xenin levels, locomotor activity and metabolic rate were also examined.

6.3 MATERIALS AND METHODS

6.3.1 Animals

Sub chronic studies were performed using male C57BL/6 mice (10-12 weeks old). All animals were maintained as described previously in Sections 2.13 and 2.13.3.

6.3.2 Experimental design

Figure 6.1 shows experimental timeline for this study. Animal model was derived as described in section 2.13.3. Treatment regimens were conducted as described in section 2.16.1. At regular intervals during the study, cumulative food and water intake, body weight, circulating glucose and insulin levels were assessed (Section 2.16.1). In addition, on day 18, locomotor activity and metabolic rate were assessed as described in Section 2.16.4. At the end of the treatment period, glucose tolerance (18 mmol/kg bw; i.p.; 18 h-fasted mice), insulin sensitivity (25 U/kg bovine insulin; i.p.; non-fasted mice) and pyruvate tolerance (2 g/kg sodium pyruvate; i.p.; 18 h-fasted mice) were assessed (Sections 2.16.2.1, 2.16.2.3 & 2.16.2.4). HOMA-IR was calculated as a surrogate marker of insulin resistance (Section 2.16.2.4). Terminal analyses included extraction of pancreatic tissue for measurement of hormone content, as well as

extraction of intestinal tissue for determination of xenin concentrations (Section 2.16.6, 2.16.7 & 2.16.9).

6.3.3 Biochemical analysis

Blood samples were collected as described in Section 2.15. Glucose and insulin were assayed as described in Sections 2.11.2 and 2.15. Assessment of GIP, GLP-1 and xenin concentrations were carried out as described in Sections 2.16.8 & 2.16.9.

6.3.4 Statistical analysis

Statistical analysis was completed using GraphPad PRISM (Version 5) as described in Section 2.18.

6.4 RESULTS

6.4.1 Effects of TNP-470, sitagliptin or a combination of both compounds on body weight, energy and fluid intake, glucose and insulin concentrations in HFF mice

Treatment for 18 days with TNP-470 alone, or in combination with sitagliptin, significantly reduced percentage body weight gain ($P<0.001$) and cumulative energy intake ($P<0.05$ - $P<0.001$) in HFF, when compared to both saline controls and sitagliptin treated mice (Figure 6.2A, B). Only TNP-470 treatment alone affected fluid intake, with significant ($P<0.01$ - $P<0.001$) reductions from day 14 onwards compared to HFF controls (Figure 6.2C). Non-fasting glucose concentrations were reduced ($P<0.05$ - $P<0.01$) in a time-dependent manner by all treatment regimens, although significant ($P<0.05$) benefits on day 4 were only evident in the combined treated group (Figure 6.3A). All treatment groups displayed progressive increases in

circulating insulin concentrations, being significantly ($P<0.05$ - $P<0.001$) elevated compared to HFF saline controls from day 11 onwards (Figure 6.3B)

6.4.2 Effects of TNP-470, sitagliptin or a combination of both compounds on glucose and pyruvate tolerance, insulin sensitivity as well as pancreatic insulin content in HFF mice

Following a glucose load, all HFF mice treated with TNP-470, sitagliptin or a combination of both drugs displayed reductions ($P<0.05$ - $P<0.001$) in individual and overall glucose concentrations (Figure 6.4A, B). Notably, overall AUC glucose in the combined treatment group were significantly ($P<0.05$) reduced compared to either treatment alone (Figure 6.4B). Corresponding glucose-induced insulin concentrations were significantly ($P<0.05$ - $P<0.01$) reduced by all treatment groups at 30 and 60 min post-injection compared to HFF controls (Figure 6.4C). Overall insulin AUC values were accordingly decreased by TNP-470 alone ($P<0.05$), and to a significantly greater degree ($P<0.05$) in the combined treatment group (Figure 6.4D). Following a pyruvate load, TNP-470 monotherapy resulted in significant ($P<0.05$ - $P<0.001$) decreases in glucose levels compared to HFF saline controls (15 and 30 min), with combined group displaying significant ($P<0.05$) reductions at 15 min post-injection (Figure 6.5A). Overall AUC glucose values were reduced ($P<0.01$ - $P<0.001$) by all treatment groups, with TNP-470 and combined treatment interventions also inducing reductions ($P<0.001$ and $P< 0.05$, respectively) when compared to sitagliptin alone (Figure 6.5B). Sitagliptin therapy alone, and in combination with TNP-470, significantly improved ($P<0.01$) peripheral insulin sensitivity following a bolus insulin injection (Figure 6.6A). Interestingly, HOMA-IR was significantly ($P<0.01$ - $P<0.001$) decreased in all HFF treatment groups when

compared to controls (Figure 6.6B), whereas pancreatic insulin content was significantly ($P<0.01$ - $P<0.001$) elevated (Figure 6.6C).

6.4.3 Effects of TNP-470, sitagliptin or a combination of both compounds on total GIP, total GLP-1 and xenin levels in HFF mice

Following 18-day treatment regimen, only the combined treatment approach resulted in significantly ($P<0.05$) elevated total plasma GIP concentrations (Figure 6.7A). However, all sitagliptin treated mice had significantly ($P<0.05$) increased total GLP-1 plasma levels on day 18 (Figure 6.7B). Plasma xenin levels were significantly ($P<0.05$ - $P<0. 01$) increased in all HFF groups of mice treated with TNP-470 (Figure 6.7C). Similar to this, only TNP-470 treatment augmented ($P<0. 01$) intestinal xenin concentrations (Figure 6.7D).

6.4.4 Effects of TNP-470, sitagliptin or a combination of both compounds on islet histology in HFF mice

Images of pancreatic islets stained for insulin and glucagon from all groups of mice are shown in Figure 6.8 A-D, with visible increases of islet area in all treatment groups. Indeed, following appropriate quantification, it was confirmed that all treatment groups had significantly ($P<0.05$ and $P<0. 01$) increased overall islet and beta cell areas when compared to HFF controls (Figure 6.8E,F). Alpha cell area was decreased ($P<0.001$) by all treatment regimens (Figure 6.8G). Consequently, beta to alpha cell ratios were significantly ($P<0.001$) increased by all treatment interventions (Figure 6.8H), with combined treatment inducing further significant ($P<0.05$) increase in beta:alpha cell ratio compared to sitagliptin therapy alone (Figure 6.8H).

6.4.5 Effects of TNP-470, sitagliptin or a combination of both compounds on O_2 consumption, CO_2 production, respiratory exchange ratio (RER), energy expenditure (EE), cumulative food intake and locomotor activity in high fat fed mice

Following 18-days treatment with TNP-470, sitagliptin or TNP-470 in combination with sitagliptin, mice (n=6) were placed in CLAMS metabolic chambers, with effects on metabolic rates and locomotor activity recorded for 21 h. None of the treatment groups displayed any significant effects on O_2 consumption or CO_2 production on day 18 (Figure 6.9 & 6.10). In addition, there were no differences between the groups of HFF mice in terms of RER during the light phase (Figure 6.11A). However, all treatments significantly ($P<0.01$) reduced RER during the dark phase (Figure 6.11B). Energy expenditure was significantly ($P<0.01$) increased by sitagliptin during the light phase, and by sitagliptin alone ($P<0.01$), and when combined with TNP-470 ($P<0.05$), during the dark phase (Figure 6.12A,B). During this 21 h CLAMS analysis, cumulative food intake was significantly reduced by TNP-470 alone and the combined treatment group (Figure 6.13A,B). There were no changes in ambulatory locomotor activity, as assessed by X-beam breaks, between groups of mice during the light phase (Figure 6.14A). However, ambulatory activity was significantly ($P<0.05$ and $P<0. 01$) increased in all treatment groups during dark phase (Figure 6.14B). Rearing events, as assessed by Z-beam breaks, were similar in both treatment and control HFF groups of mice during light phase (Figure 6.14C). Treatment with TNP-470 alone, or in combination with sitagliptin, significantly ($P<0.05$ - $P<0.01$) reduced Z-beam breaks during dark phase (Figure 6.14D).

6.5 DISCUSSION

The primary mechanism of action of DPP-4 inhibitors is augmentation of the incretin effect, known to be severely compromised in patients with T2DM [Knop *et al*. 2007b]. Whilst elevation of circulating GLP-1 concentrations by DPP-4 inhibitors has established benefits in T2DM [Gallwitz, 2007], there is a well-characterised insensitivity to the biological actions of GIP in this disease [Vilsbøll *et al*. 2002]. Thus, incorporating strategies to enhance GIP bioactivity when administering DPP-4 inhibitors could represent an extremely attractive therapeutic strategy. Such an approach holds even more merit given observations of significant complimentary beneficial antidiabetic actions of GIP and GLP-1 in T2DM patients [Portron *et al*. 2017; Frias *et al*. 2017, 2018]. Based on this premise, and the promising results displayed in chapter 5, the present study has integrated DPP-4 and MetAP2 inhibition, utilising established knowledge that xenin can potentiate the biological actions of GIP [Wice *et al*. 2010, 2012; Taylor *et al*. 2010; Chowdhury *et al*. 2014; Martin *et al*. 2014], together with the likelihood that part of the antidiabetic benefits of MetAP2 inhibitors are linked to inhibition of xenin degradation.

Treatment with sitagliptin or TNP-470 alone induced various metabolic benefits in HFF mice. Such improvements included substantially reduced glucose concentrations, as well as improved glucose and pyruvate tolerance in addition to increased plasma and pancreatic insulin concentrations. These metabolic benefits were associated with notable improvements in islet morphology, which is particularly noteworthy given that the HFF mice were also administered STZ, a recognised beta cell toxin [Lenzen, 2008]. Positive effects are even more encouraging considering the somewhat reduced doses of sitagliptin and TNP-470 we employed [White *et al*. 2012; Gault *et al*. 2015b].

Indeed, although expected [Gault *et al*. 2015b], treatment with sitagliptin alone increased circulating GLP-1 levels, surprisingly corresponding plasma GIP levels were not significantly elevated in these mice. This could imply that at the 25 mg/kg dose of sitagliptin used, antidiabetic benefits are largely linked to augmentation of GLP-1 actions. However, investigation of plasma levels of other DPP-4 substrates with a potential impact upon metabolism, such as neuropeptide Y or Peptide YY [Khan *et al*. 2017b; Lafferty *et al*. 2018a; 2019], would also be necessary to confirm this, but outside the scope of the current study. Remarkably, even at the relatively reduced dose of 1 mg/kg, TNP-470 clearly augmented both plasma and intestinal xenin concentrations, whether administered alone or in combination with sitagliptin. Thus, metabolic benefits of MetAP2 inhibition do appear to be mediated, at least in part, by enhanced xenin activity [Gault *et al*. 2015a; Martin *et al*. 2014, 2016; Craig *et al*. 2018].

In addition to elevated plasma GLP-1 and xenin concentrations in the combined sitagliptin and TNP-470 treatment group, these mice also presented with notably increased GIP levels. In this regard, the secretory dynamics of intestinal K-cells has previously been explored in depth [Gribble and Reimann, 2019], but whether xenin can further augment GIP secretion still needs to be determined. Nonetheless, combined upregulation of GIP, GLP-1 and xenin signalling pathways was associated with distinct benefits over either treatment option alone. This involved a more rapid restoration of normoglycaemia and dramatically improved glucose tolerance. Interestingly, benefits on glucose disposal were in conjunction with a significantly decreased, as opposed to increased, glucose stimulated insulin secretory profile. This being despite the established notable beta cell stimulatory effects of GIP, GLP-1 and

xenin [Seino *et al*. 2010; Craig *et al*. 2018], and these mice having a much improved beta:alpha cell ratio. Hepatic gluconeogenesis was not improved in the combined treatment group, as determined through assessment of pyruvate tolerance, implying improved glycaemic status is linked to enhanced insulin action in these mice. This was indeed manifest during insulin tolerance tests, although it is worthwhile noting that such a method of assessment of insulin action is limited by normal adaptive responses to prevent glucose concentrations falling towards life threatening hypoglycaemic levels. To account for this, we also calculated HOMA-IR [Wallace *et al*. 2004] that again evidenced clear improvements in insulin resistance. Reductions in body weight could be one explanation for improved insulin sensitivity in the treatment groups [Clamp *et al*. 2017], but unlikely given that sitagliptin therapy did not induce weight loss.

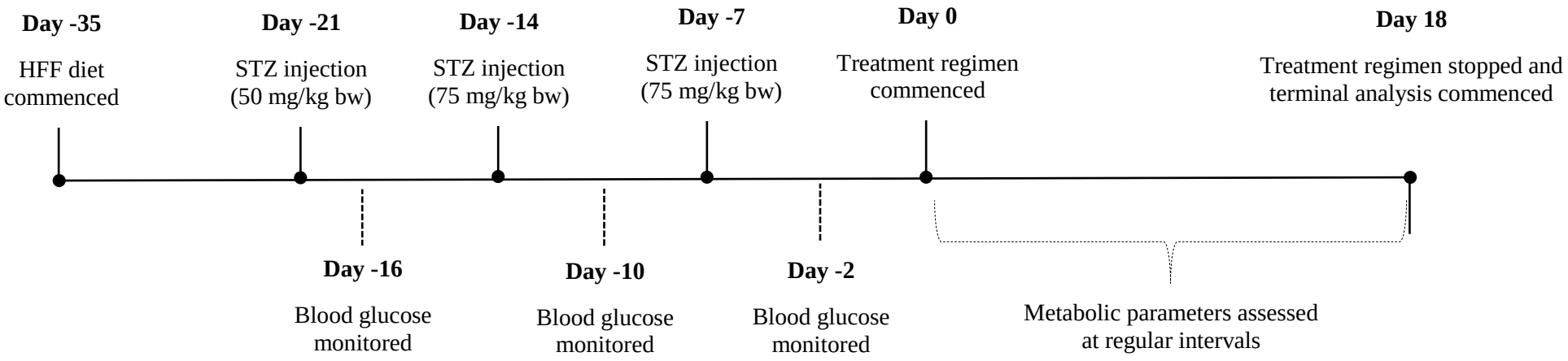
In agreement with previous reports [Rupnick *et al*. 2002; White *et al*. 2012], all TNP-470 treated mice had reduced body weight gain, that was associated with appetite suppressive actions. In harmony with this, xenin has previously been documented to possess anorectic effects [Taylor *et al*. 2010]. In addition, in accordance with the elevated GIP and GLP-1 levels in the combined treatment group, robust reductions of appetite and body weight have been evidenced following sustained upregulation of GIP and GLP-1 signalling in rodents [NamKoong *et al*. 2017] and humans [Coskun *et al*. 2018]. In contrast, whilst GLP-1 infusion has recently been shown to lower energy intake in man, simultaneous GIP infusion did not potentiate this effect [Bergmann *et al*. 2019]. Thus, to further investigate potential related mechanisms, metabolic rate and locomotor activity were assessed at the end of the treatment cycle. As expected, given the prominent effects of GIP, GLP-1 and xenin on energy balance

[Baggio and Drucker, 2007; Barrera *et al*. 2011; Craig *et al*. 2018], all treated mice had decreased respiratory exchange ratio, with sitagliptin treatment evoking a further increase in energy expenditure. Since TNP-470 induces appetite suppression, and the impact of sitagliptin on markers of energy turnover, it is perhaps surprising that a more striking benefit on body weight loss was not observed in the combined treatment group. However, DPP-4 inhibitors are well recognised to have body weight neutral actions [Gallwitz, 2007], and all mice had similar increases of locomotor activity. Thus, assessment of tissue thermogenic capacity may be useful to uncover related mechanisms. Indeed, GIP, GLP-1 and xenin have all been revealed to alter lipid metabolism and adipocyte function [McClean *et al*. 2007; Irwin and Flatt, 2009; Vendrell *et al*. 2011; Farr *et al*. 2014; Bhavya *et al*. 2017]. In addition, these observations on body weight control could simply be a reflection of the plasticity in signalling pathways linked to overall energy homeostasis [Lenard and Berthoud, 2008]. As such, metabolic rate in diabetic mice was unaltered by sustained dual activation of GLP-1 and xenin pathways [Hasib *et al*. 2018a]. Moreover, long-acting GLP-1 analogues are believed to modestly reduce locomotor activity [Sorensen *et al*. 2015], whereas related GIP [Gault *et al*. 2011b] or xenin [Gault *et al*. 2015a] analogues have been shown to increase this parameter.

Clinical application of MetAP2 inhibitors has been limited due to their high toxicity profiles [Chang, 2017]. In contrast to this, previous studies using TNP-470 has revealed a relatively tolerable toxicity profile [Kruger and Figg, 2000; Bråkenhielm *et al*. 2004]. It should be emphasised that mice treated with TNP-470 did not alter animal behaviour. However, a more thorough examination of toxicity at the organ / tissue level is still needed, but initial observations are positive.

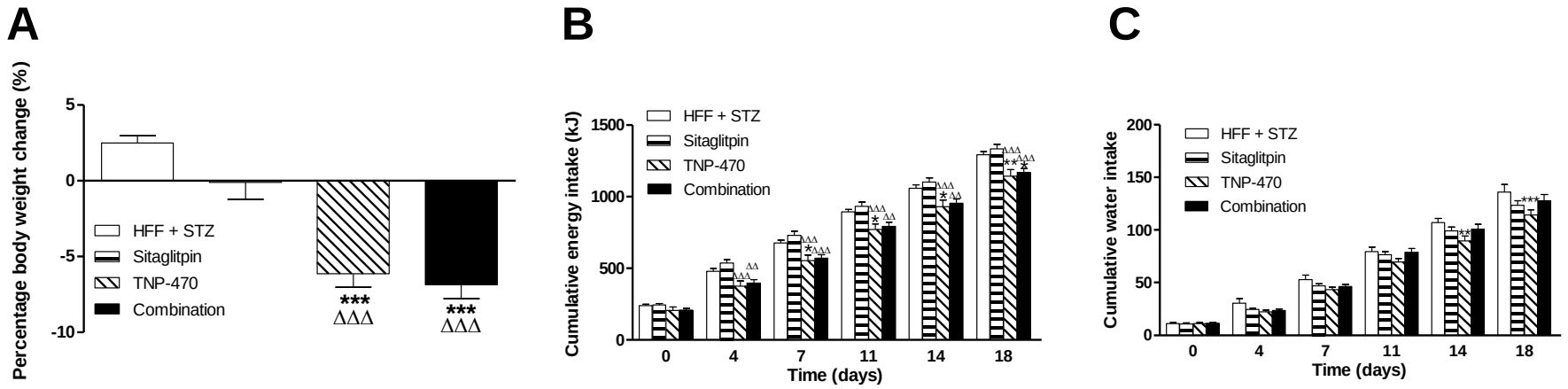
In conclusion, the current study establishes that the antidiabetic efficacy of DPP-4 inhibition can be augmented by concurrent use of MetAP2 inhibitors, such as TNP-470. These benefits appear to be, at least in part, linked to an elevation of xenin concentrations by TNP-470, and the recognised ability of xenin to potentiate the biological actions of GIP [Taylor *et al*. 2010; Martin *et al*. 2012, 2014; Gault *et al*. 2015a; Parthsarathy *et al*. 2016]. Taken together, such an approach merits further investigation as a potential novel treatment option for T2DM.

Figure 6.1 Timeline for experimental study



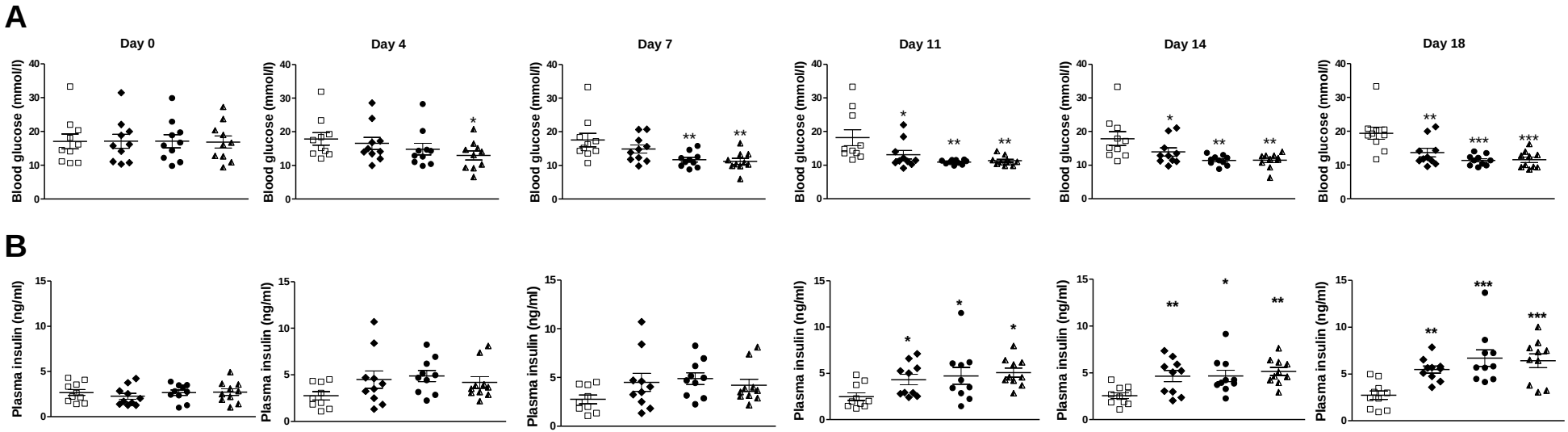
Streptozotocin (STZ) treatment: Three low dose STZ injections (50 mg/kg bw, 75 mg/kg bw & 75 mg/kg bw, i.p.) at 1- week intervals
Treatments started on Day 0;
Group 1: HFF control – HFF saline vehicle (0.9% w/v NaCl, i.p. / oral) for 18 days
Group 2: Sitagliptin – HFF Sitagliptin (25 mg/kg bw, oral) for 18-days
Group 3: TNP-470 – HFF TNP-470 (1 mg/kg bw, i.p.) for 18-days
Group 4: Sitagliptin + TNP-470 – HFF Sitagliptin (25 mg/kg bw, oral) + TNP-470 (1 mg/kg bw, i.p.) for 18-days

Figure 6.2 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on (A) body weight, (B) energy intake and (C) water intake.



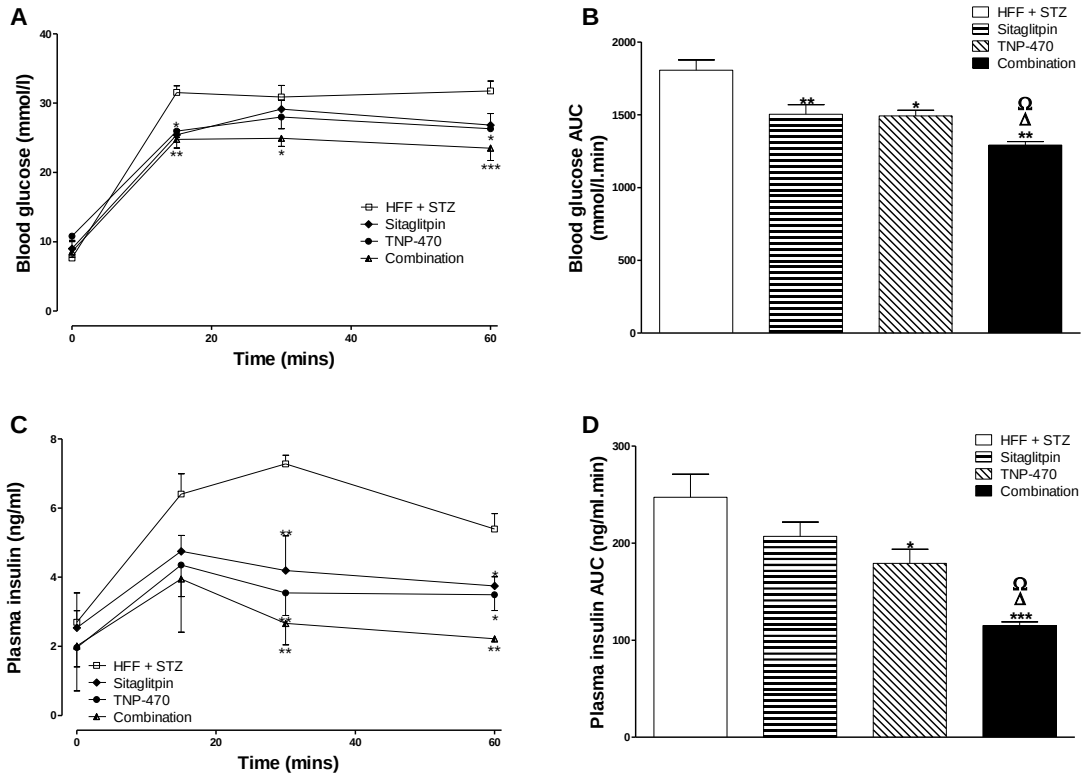
Parameters were measured during 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw. i.p. once daily) or a combination of both compounds. Values are mean ± SEM for ten mice. *P<0.05, **P<0.01 and ***P<0.001 compared to HFF saline controls. $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to sitagliptin alone.

Figure 6.3 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on (A) glucose and (B) insulin concentrations in HFF mice.



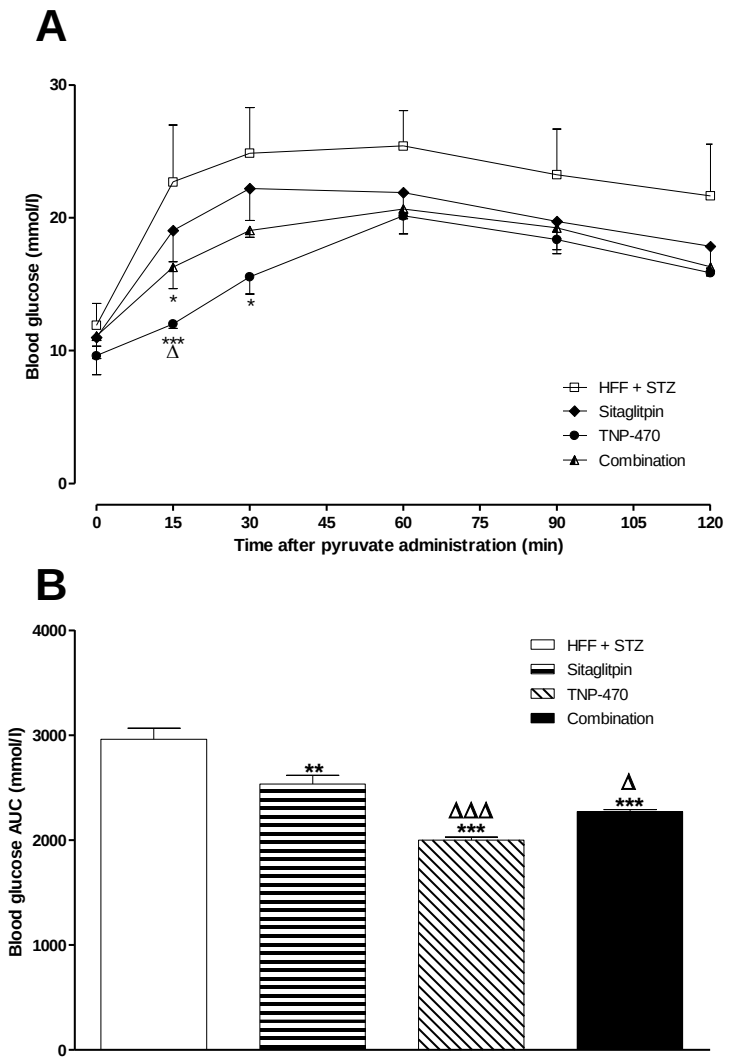
Parameters were measured during 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw. i.p. once daily) or a combination of both compounds. Values are mean ± SEM for ten mice. *P<0.05, **P<0.01 and ***P<0.001 compared to HFF saline controls.

Figure 6.4 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on glucose tolerance and insulin secretion.



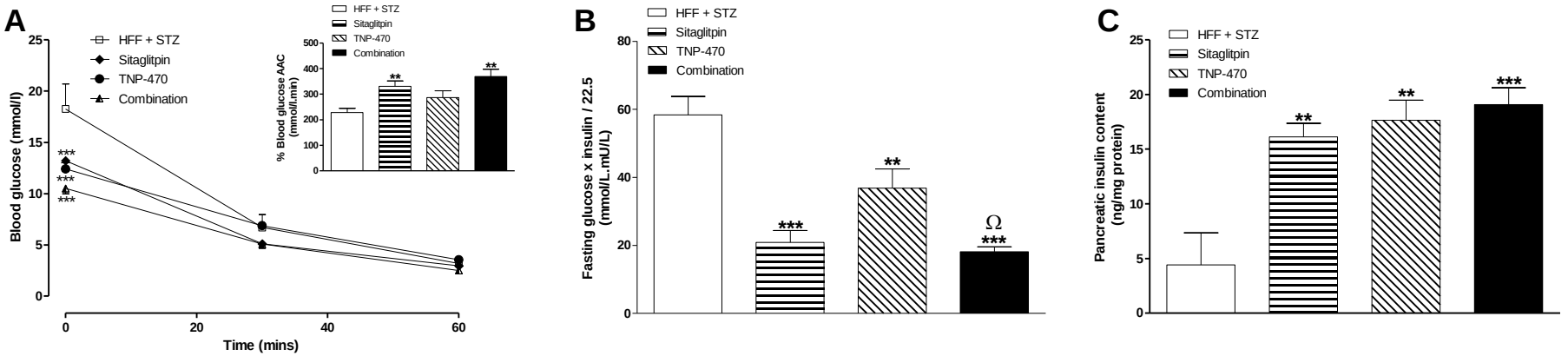
Parameters were assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw. i.p. once daily) or a combination of both compounds using the same dosing regimens. (A,C) Glucose (18 mmol/kg bw) was administered by i.p. injection at t=0 min in 18 hour fasted mice. (B,D) Glucose and insulin AUC values for 0–60 min post injection. Values are mean ± SEM for ten mice. *P<0.05, **P<0.01 and ***P<0.001 compared to HFF saline controls. $^{\Delta}$P<0.05 compared to sitagliptin alone. $^{\Omega}$P<0.05 compared to TNP-470 alone.

Figure 6.5 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on pyruvate tolerance in HFF mice.



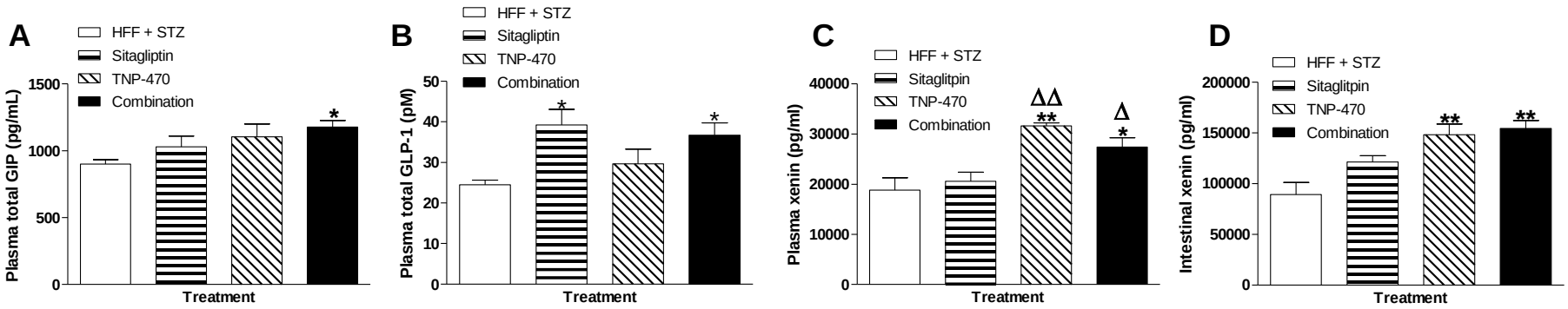
Pyruvate (2 g/kg bw, i.p.) at t=0 min in 18 hour fasted mice (A). Blood glucose AUC values for 0–120 min post injection (B). Values are mean ± SEM for ten mice. *P<0.05, **P<0.01 and ***P<0.001 compared to HFF saline controls. $^{\Delta}$P<0.05 and $^{\Delta\Delta\Delta}$P<0.001 compared to sitagliptin alone.

Figure 6.6 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on insulin sensitivity and pancreatic insulin content in HFF mice.



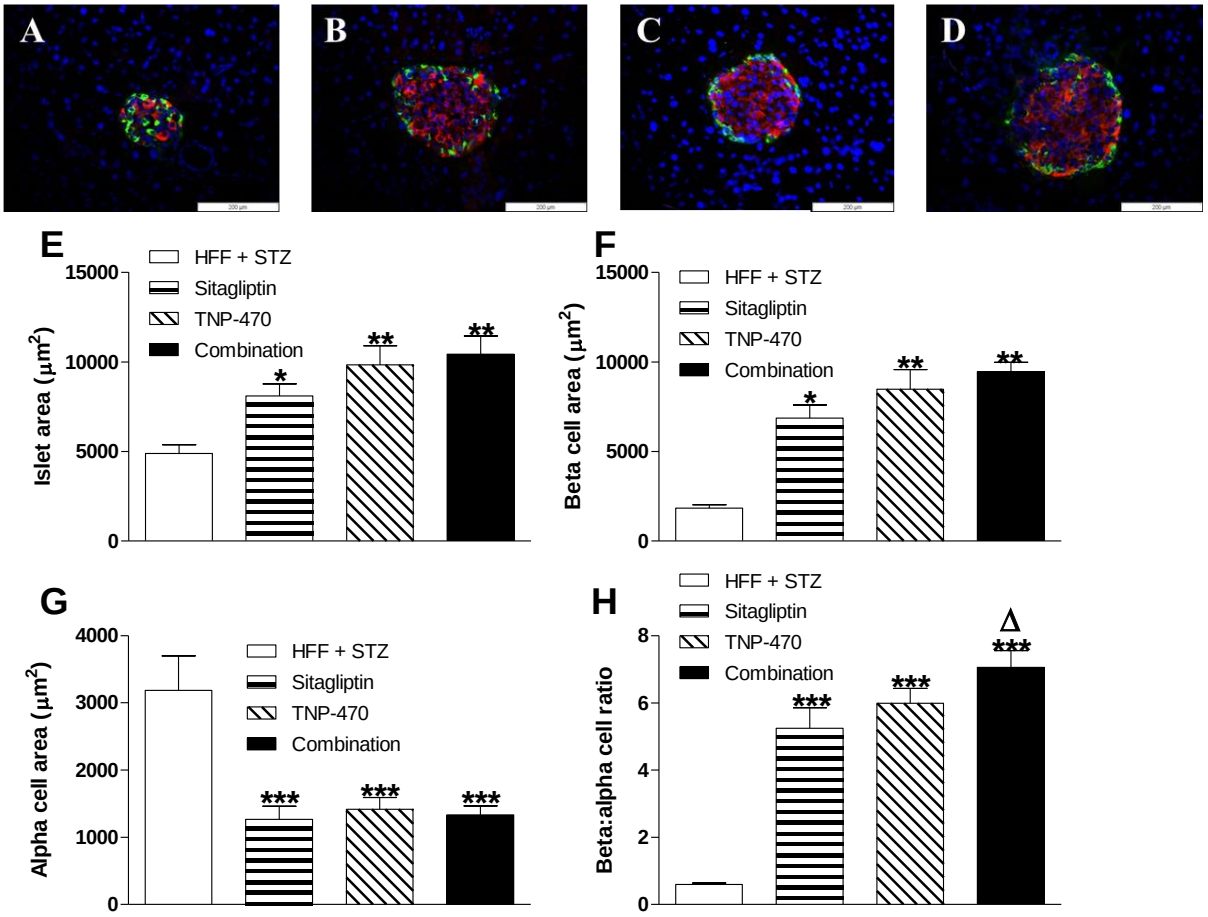
Parameters were assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds using the same dosing regimens. (A) Insulin (25 U/kg bw) was administered by i.p. injection at t=0 min in non-fasted mice. Glucose AAC values post injection are shown. (B) HOMA-IR was calculated by the following equation: fasting glucose (mmol/L) x fasting insulin (mU/L) / 22.5. (C) Pancreatic insulin content was measured by RIA following acid-ethanol extraction. Values are mean ± SEM for ten mice. **P<0.01 and ***P<0.001 compared to HFF saline controls. $^{\Omega}$P<0.05 compared to TNP-470 alone.

Figure 6.7 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on total plasma GIP and GLP-1, as well as plasma and intestinal xenin levels.



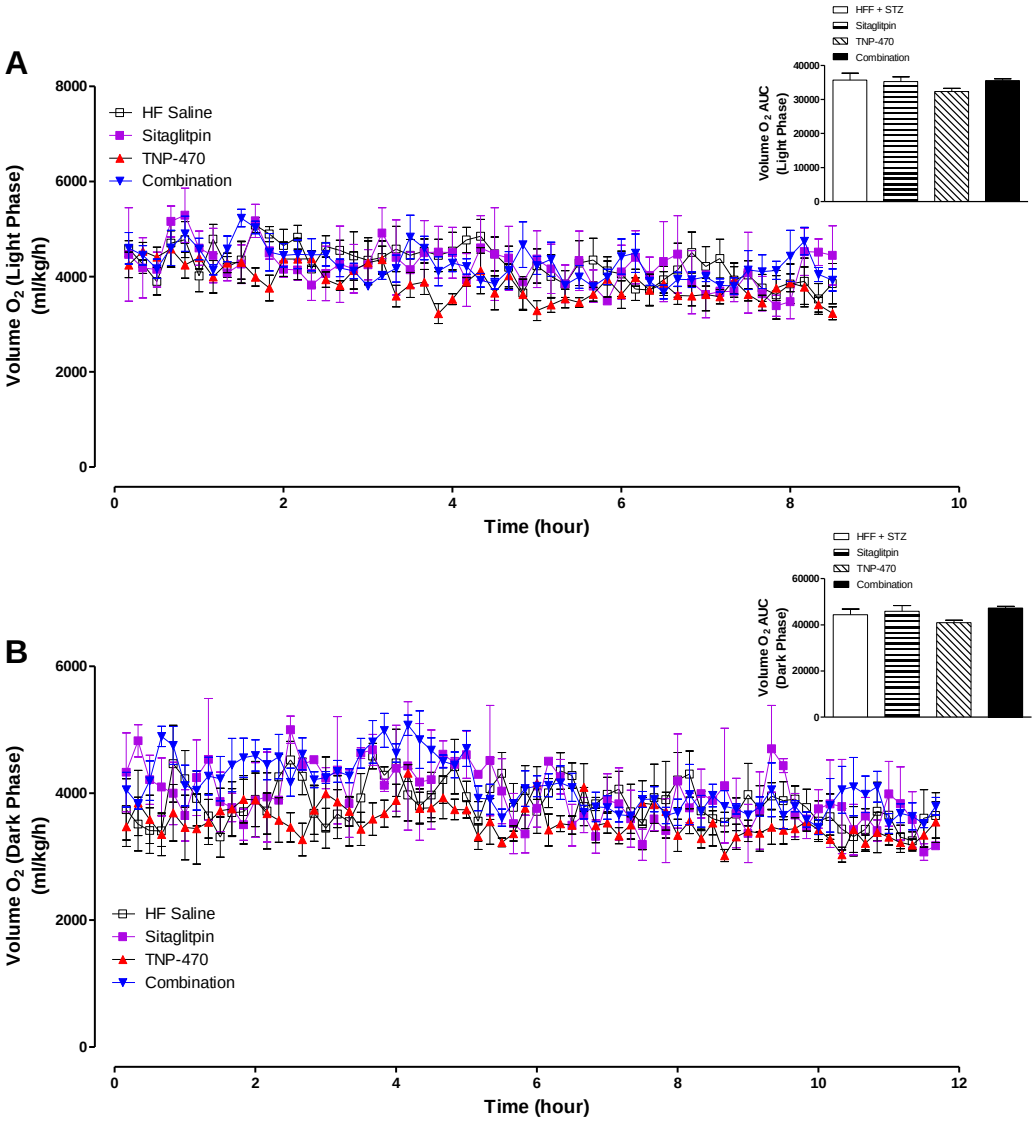
Parameters were assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw. i.p. once daily) or a combination of both compounds using the same dosing regimens. (A) Total plasma GIP, (B) total plasma GLP-1, (C) plasma xenin and (D) intestinal xenin concentrations were assessed using ELISAs. Values are mean ± SEM (A,B; n= 10, C,D; n=6) *P<0.05 and **P<0.01 compared to HFF saline controls. $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 compared to sitagliptin alone.

Figure 6.8 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on pancreatic islet architecture in HFF mice.



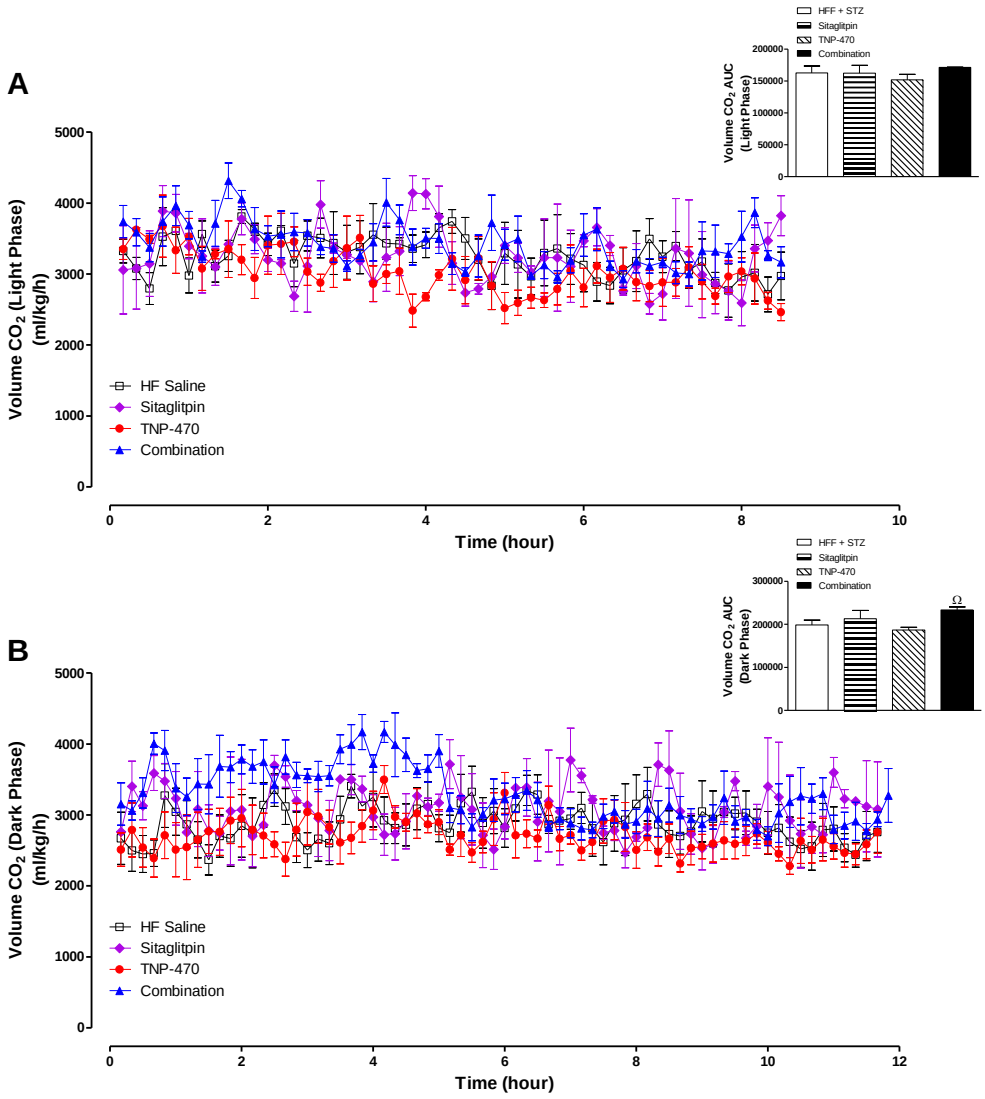
Parameters were assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds. (A-D) Representative images (20X) of islets showing insulin (red) and glucagon (green) immunoreactivity from each treatment group. (E) Islet, (F) beta- and (G) alpha-cell areas, as well as (H) beta:alpha area cell area ratio were measured using Cell^F image analysis software. Values are mean ± SEM for ten mice. *P<0.05, **P<0.01 and ***P<0.001 compared to HFF saline controls. $^{\Delta}$P<0.05 compared to sitagliptin alone.

Figure 6.9 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on O_2 consumption in HFF mice.



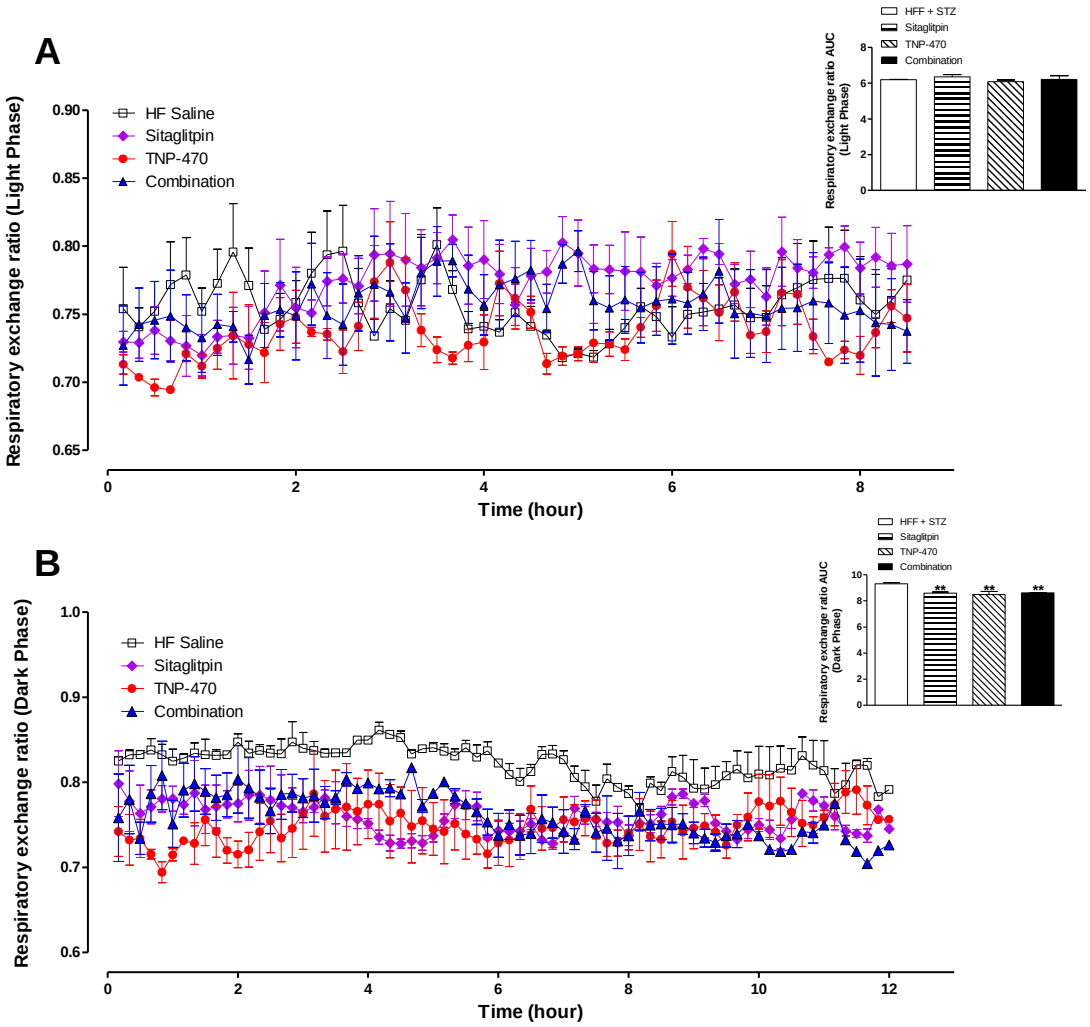
O_2 consumption was assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds using the same dosing regimens. Mice were placed in CLAMS metabolic chambers for 21 h. Values represent mean ± S.E.M. for 6 mice.

Figure 6.10 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on CO_2 production in HFF mice.



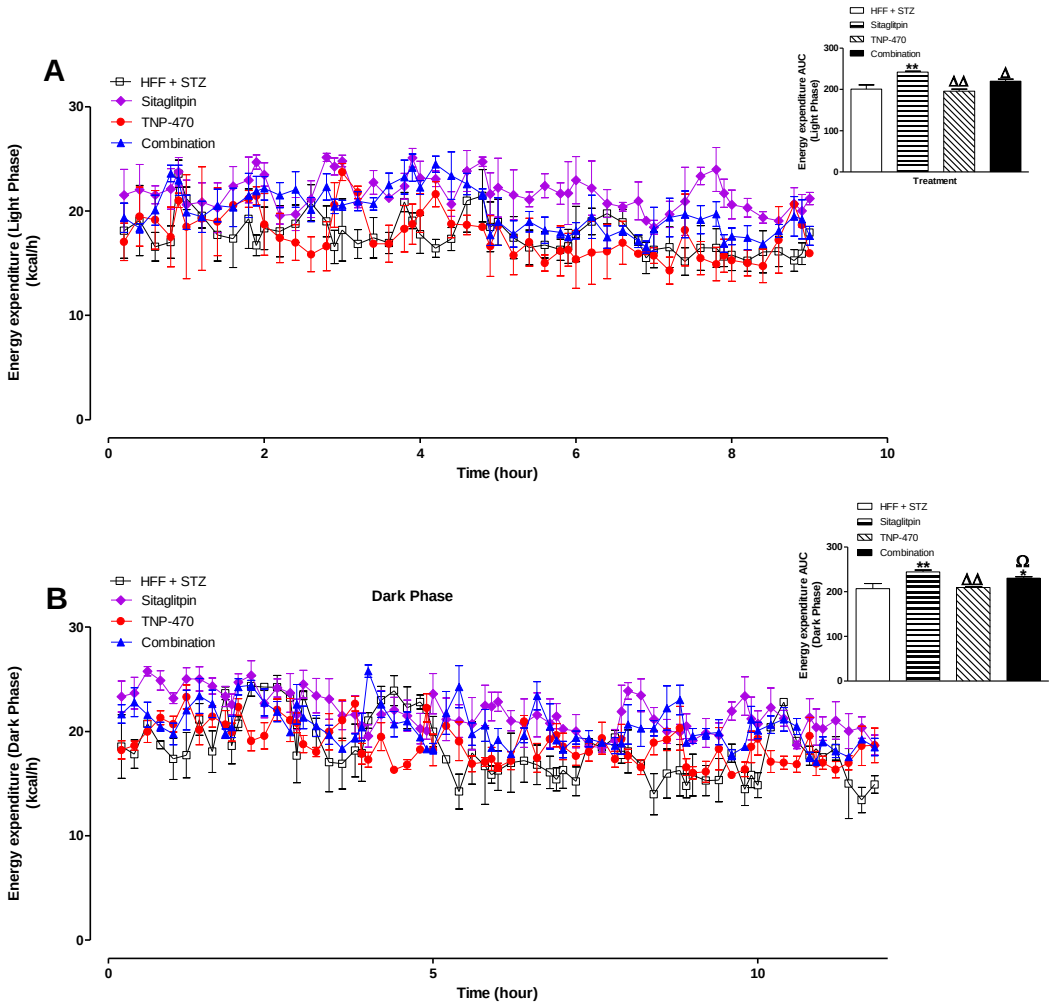
CO_2 production was assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds using the same dosing regimens. Mice were placed in CLAMS metabolic chambers for 21 h. Values represent mean ± S.E.M. for 6 mice. $^{\Omega}P<0.05$ compared to TNP-470 alone.

Figure 6.11 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on respiratory exchange ratio (RER) in HFF mice.



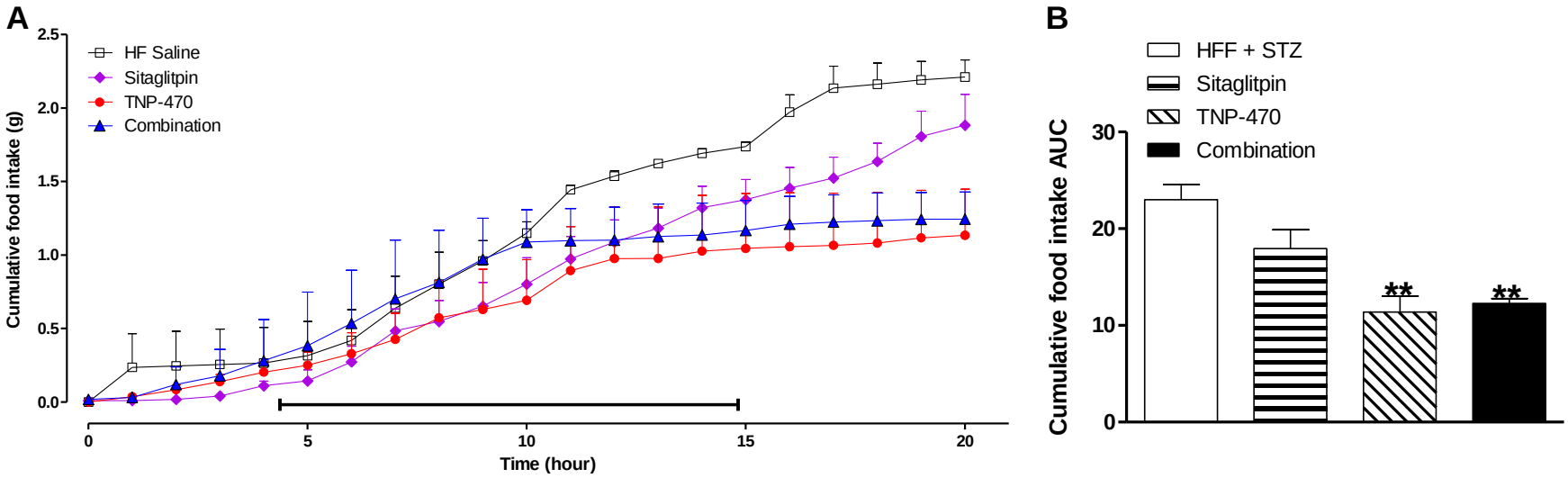
RER was assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds using the same dosing regimens. Mice were placed in CLAMS metabolic chambers for 21 h and RER during the (A) light and (B) dark phases assessed, with RER AUC displayed in the inset. Values represent mean ± S.E.M. for 6 mice. **P<0.01 compared to HFF saline controls.

Figure 6.12 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on energy expenditure (EE) in HFF mice.



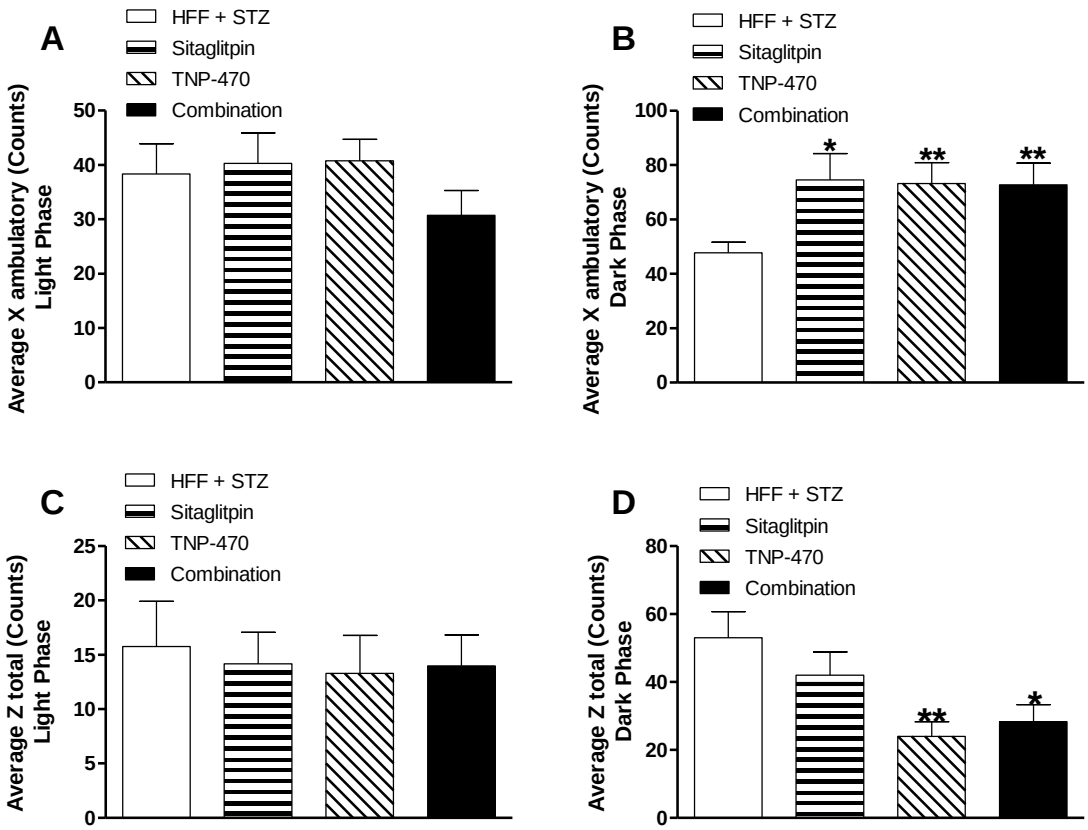
Energy expenditure was assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds using the same dosing regimens. Mice were placed in CLAMS metabolic chambers for 21 h, with energy expenditure (EE), during the (A) light and (B) dark phases, measured. Values represent mean ± S.E.M. for 6 mice. *P<0.05 and **P<0.01 compared to HFF saline controls. $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 compared to sitagliptin alone. $^{\Omega}$P<0.05 compared to TNP-470 alone.

Figure 6.13 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on cumulative food intake in HFF mice.



Cumulative food intake was assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds using the same dosing regimens. Mice were placed in CLAMS metabolic chambers for 21 h, with cumulative food intake measured. Values represent mean ± S.E.M. for 6 mice. **P<0.01 compared to HFF saline controls.

Figure 6.14 Effects of 18-day treatment with TNP-470, sitagliptin or a combination of both compounds on locomotor activity in HFF mice.



Locomotor activity was assessed following 18-day treatment with sitagliptin (25 mg/kg bw, p.o., once daily), TNP-470 (1 mg/kg bw, i.p., once daily) or a combination of both compounds using the same dosing regimens. Mice were placed in CLAMS metabolic chambers for 21 h. Activity counts in the (A, B) X- and (C, D) Z- axes were recorded every minute for 21 h. Values represent mean ± S.E.M. for 6 mice. *P<0.05 and **P<0.01 compared to HFF saline controls.

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Chapter 7

General Discussion

7.1 Type 2 diabetes mellitus – an increasing epidemic

T2DM, the most common type of diabetes, is now considered a major global health problem, due to its rapidly increasing prevalence and economic burden it imposes. Characterised by both beta cell dysfunction and insulin resistance, T2DM is a complex metabolic disorder that is closely related to increasing prevalence of obesity [Ozcan *et al*. 2004; Malone and Hansen, 2019]. T2DM, caused by the decreased response and utilisation of insulin by peripheral tissues, coupled with the insensitivity of beta cells, is progressed through the escalation of both insulin insensitivity and deficiency. T2DM and its associated micro- and macro-vascular complications [Fowler, 2008], contribute to the multi-morbidity and mortality associated with this disease, thereby creating a huge economic burden, and therefore necessitating the need for novel, more effective treatment paradigms.

7.2 Limitations of current anti-diabetic therapeutics

First and foremost, life-style changes, including a healthy diet and exercise regime, is recommended to achieve normoglycaemia. However, this often fails leading to pharmacological intervention being required. There is a plethora of anti-diabetic therapies available, with the key aim being the maintenance of normal glucose homeostasis and preservation of beta cell function, whilst attenuating associated micro- and macro-vascular complications. First line monotherapy routinely fails to achieve and maintain normoglycaemia, with long-term patient response rates deteriorating over time, necessitating the need for combinational therapy approaches to maintain glycaemic control. Nevertheless, these current therapeutics fail to combat chronic complications. In addition, due to the complex, multifactorial pathophysiology of this disease and the multiple organs it can affect, current therapies that only exploit

a single glucoregulatory pathway, such as insulin secretion or insulin insensitivity [Bailey, 2000], fail to achieve the desired prolonged response. Treatments also are inadequate in restoring beta cell mass and impaired beta cell function that accompanies T2DM, which ultimately leads to beta cell exhaustion and worsens prognosis [Bailey, 2000]. Therefore, an efficient T2DM therapy should aim to reverse the major pathophysiological abnormalities associated with this disease, including impaired insulin secretion, incretin deficiency, enhanced glucagon secretion, hepatic glucose production, accelerated lipolysis in adipocytes, increased glucose reabsorption in the kidney as well as decreased uptake in muscle [De Fronzo *et al*. 2014]. Hence, novel anti-diabetic agents, which have multiple exploitation capacities, are required to sustain improved management and decrease the associated complications.

According to recent ADA/EASD guidelines, metformin monotherapy continues to be the first-line recommended therapy for almost all patients with T2DM, however, 20-30% of patients have suffered metformin-associated adverse effects, including gastrointestinal tract discomfort, nausea and vomiting, with approximately 5% of patients unable to tolerate metformin at all [Dujic *et al*. 2015; McCreight *et al*. 2016]. Metformin is followed by addition of second-line glucose-lowering medications, such as sulfonylureas, DPP-4 inhibitors or thiazolidinediones, however the efficacy of these are unsustainable due to undesirable side effects which promote weight gain and increase hypoglycaemic risk [Kerru *et al*. 2018]. The intensification of treatments beyond dual-therapy, which is more often required, increases side-effects as well as amplifying the burden and cost of treatment. Therefore, different treatment models need to be implemented to achieve optimum cost-effective approaches to both diabetic care and management, as well as sustained glycaemic control.

More recent advances in therapies for T2DM has identified incretin mimetics as a promising drug class. These mimetics, with increased biological stability, have shown promise in the maintenance of glucose homeostasis and beneficial effects on beta cells [Drucker, 2006; Irwin and Flatt, 2015]. Incretin mimetics have also been shown to omit the risk of hypoglycaemia [Hansen *et al*. 2010], with other favourable effects on both cardio health and protection [Hansen *et al*. 2010], as well as benefits on cognitive function [Porter *et al*. 2011]. However, incretin mimetics focus on augmenting the actions of the incretin GLP-1, with much less attention on promoting the biological actions of GIP, which has been designated as the major incretin hormone, accounting for 70% of the incretin effect [Gault *et al.* 2003c; Holst, 2019]. Therefore, the potentiation of this incretin is missing in current therapies and therefore novel therapeutics with GIP potentiating actions is warranted.

7.3 Therapeutic application of gut derived peptides

There has been considerable interest in bioactive peptides as a novel drug class, due to favourable anti-diabetic, anti-cancerous and anti-microbial properties [Yan *et al*. 2019]. For treatment of diabetes, peptide hormones released from the intestine act as key regulators in postprandial insulin release and energy regulation [Drucker, 2007; Monteiro and Batterham, 2017]. Receptors for these gut hormones are widely distributed throughout the body in organs including the pancreas, brain, muscle and adipose tissue [Usdin *et al*. 1993; Holst, 2007; Baggio and Drucker, 2007], and in turn play vital roles in glucose and energy homeostasis. The incretin hormones, GLP-1 and GIP, are the two most studied gut hormones in diabetes and are primarily involved in glucose-stimulated insulin secretion [Seino *et al*. 2010]. Further research has also

highlighted beneficial extrapancreatic effects and favourable metabolic profiles, in addition to the glucose-lowering incretin mediated effect [Yamada *et al*. 2016]. However, they are rapidly degraded by the enzyme DPP-4. For clinical exploitation, stable, long-acting analogues of GIP have been generated [Irwin *et al*. 2006b; Kerr *et al*. 2009; Tatarkiewicz *et al*. 2014], displaying anti-diabetic promise, however, have not yet been clinically approved. In contrast, several GLP-1 agonists, namely liraglutide and exendin-4, have been clinically approved for therapeutic use [Eng *et al*. 1992; Gupta, 2013]. Further to this, other gut-derived hormones, including CCK, PYY and xenin, have been the focus of recent research as potential therapeutic targets alone or in combination for the treatment of T2DM [Pathak *et al*. 2018; Lafferty *et al*. 2018b; Craig *et al*. 2018]. For novel therapies aimed at managing diabetes, there has been an increase in gut-derived hormone pharmacological strategies, due to modification enabling longer-acting analogues, enhanced efficacy through combination, as well as the knowledge that the significant benefits following bariatric surgery is mainly linked to the altered secretion of various gut hormones [Pournaras *et al*. 2010].

7.4 Potentiating the biological actions of GIP

There is a misconception that GLP-1 is a superior pharmacological target for antidiabetic therapy over GIP [Bastin and Andreelli, 2019], with current therapies focusing on augmentation of GLP-1. However, GIP acts to stimulate glucose-dependent insulin secretion through the incretin effect, whilst also playing other key roles [Seino *et al*. 2010]. These additional roles include promoting beta cell proliferation and inhibiting apoptosis, thus resulting in expansion of beta cell mass, which is perturbed in T2DM. It has been suggested that GIP may regulate beta cell

survival though signalling pathways, which are independent of GLP-1, therefore proposing that both incretin hormones may have individual specific roles in T2DM therapy and may act to complement each other [Campbell *et al*. 2016]. GIP also modulates postprandial glucagon responses, whilst in addition facilitating fat deposition in adipose tissue and stimulating bone formation. Furthermore, GIP has been shown to regulate appetite and satiety effects in the brain [Seino *et al*. 2010]. However, in T2DM there is decreased secretion of GLP-1 and defective responsiveness to GIP, resulting in a diminished incretin effect, which is a key pathophysiological defect in T2DM [Knop *et al*. 2007b]. Many agents have been clinically approved to increase GLP-1 levels, however promising GIP analogues that act to augment the biological actions of GIP still await pre-clinical trials. GIP desensitisation is thought to be a result of the downregulation of GIP receptors on beta cells as a result of hyperglycaemic status in T2DM patients. However, Højberg *et al*. 2009 demonstrated that normalisation of blood glucose levels in diabetic subjects resulted in an enhanced incretin response to GIP, potentially due to up-regulation of GIP receptors on beta cells. This therefore suggests that GIP desensitisation is potentially just a consequence of the hyperglycaemic state, and that sensitivity to GIP can be restored with improved glycaemic control. Issues regarding receptor desensitisation have also been suggested as a problem with GIP based therapies, however *in vitro* glucose tolerance tests performed in chapter 3 may help to exclude this problem. Thus, GIP may not be as inconsequential as first thought in T2DM.

Further to this, the gut derived peptide hormone xenin, co-secreted from the same intestinal K-cells as GIP, potentiates the diminished biological actions of GIP in diabetes [Wice *et al*. 2010, 2012; Martin *et al*. 2012, 2014; Gault *et al*. 2015; Craig *et*

al. 2018]. Moreover, the independent insulinotropic actions of xenin, combined with its GIP augmenting abilities, have been solidified in this thesis. The work contained here has developed novel treatment paradigms, based around the gut peptide xenin, either alone, in combination or as part of a hybrid peptide to target the impaired GIP arm of the incretin axis, improving glycaemic status in various mouse models of obesity / diabetes. This represents proof-of-concept for the efficacy of xenin based therapies for diabetes.

7.5 A GIP/xenin hybrid in combination with exendin-4 as a persistent therapeutic agent

In T2DM, elevated glucose concentrations lead to desensitisation of GIP receptors, which is established as a fundamental determinant of beta-cell insensitivity to GIP causing GIP resistance [Xu *et al*. 2007]. However, this can be reversed and normal incretin action restored through improved glycaemic control [Højberg *et al*. 2009]. However, other studies have suggested that additional mechanisms are involved in the defective actions of GIP in T2DM, such as genetic defects in GIP signalling [Meier and Nauck, 2010], with Pathak *et al*. [2014] also suggesting that the defective insulinotropic actions of GIP are related to mechanisms beyond receptor expression. Previous studies within our research group have shown that the palmitate-derivatised analogue of xenin improves the biological actions of GIP [Martin *et al*. 2012, Gault *et al*. 2015a], along with previous studies showing the metabolic benefits of this analogue incorporated into a GIP hybrid molecule [Hasib *et al*. 2016]. Based on previous studies which show that combined activation of GIP and GLP-1 receptor pathways may provide additive beneficial effects compared to GLP-1 analogues alone, and following on from previous research within our laboratory, the key rationale in chapter 3 was to

assess if the already established effects of (DAla^2)GIP/xenin-8-Gln could be augmented through combination therapy with the clinically approved GLP-1 mimetic, exendin-4, and to evaluate persistent efficacy in HFF mice.

The persistent improvements in glycaemic control induced by this hybrid in combination with exendin-4 where coupled with favourable changes in body weight, but independent of any changes in energy intake. This is intriguing since appetite suppressive effects of xenin have been established, mediated through the hypothalamus, but independent of hypothalamic ERK1/2 [Kim *et al*. 2016] or melanocortin signalling pathways [Leckstorm *et al*. 2009]. In terms of body weight, treatments that elicit reductions in body weight represent a clear advantage over currently available drug classes such as thiazolidinediones or sulfonylureas, which are linked with weight gain [Cheng and Kashyap, 2011]. Exendin-4 has established weight reducing effects [Kanoski *et al*. 2016], with other suggestions of the weight reducing effects of xenin [Craig *et al*. 2018]. In this regard, further studies examining metabolic rate, locomotor activity and energy expenditure would also be useful.

Indeed, twice-daily administration also significantly improved glucose tolerance in HFF mice, with this effect persistent in nature, in all treatment groups, further confirming the therapeutic potential of this treatment paradigm in overcoming beta-cell associated problems in this HFF model. Both exendin-4 and the combination group improved insulin sensitivity, however, (DAla^2)GIP/xenin-8-Gln alone failed to improve insulin sensitivity. These improved persistent benefits on metabolic profile could be a result of enhance glycaemic control, or improved incretin hormone actions which improve both beta cell function and insulin resistance [Chon and Gautier, 2016].

Furthermore, reduction in HbA1c levels was also indicative of sustained glycaemic control, which is highly favourable in such a multifactorial disease. Thus, this durable effect of this hybrid could be due to enhanced incretin effect through augmentation of GIP actions. Future studies to assess gene and protein expression involved in synthesis or secretion of insulin as well as genes involved in beta cell proliferation and apoptosis, could help explain the persistent beneficial effects shown. Surprisingly, a lack of additive effects when combined with exendin-4 was apparent in this study. This could be a result of the good efficacy of the GIP/xenin hybrid peptide alone [Hasib *et al.* 2017], or the relatively mild form of diabetes in this model.

The combination approach employed resulted in stimulation of GLP-1, GIP and xenin signalling pathways. By augmenting all three pathways, this would help to lessen progression of disease severity. Progression of T2DM results in reduction of islet beta-cell mass, beta cell function, or both, therefore due to the favourable effects GIP, GLP-1 and xenin have on beta cells [Chon and Gautier, 2016; Craig *et al*. 2018], this combinational approach could be very favourable when disease severity progresses. As was apparent in this study, the diabetic state was progressing and worsening, illustrated by the reduced pancreatic content on day 42 compared to day 28. However, as this combined modulation may also work through similar or complementary mechanisms, thus it could preclude additive effects. Indeed, a triple-acting hybrid peptide consisting of GIP/xenin/exendin-4 may be more patient favourable through the stimulation of multiple pathways through one molecule, but unfortunately due to the hybrid structure and main bioactive region sites of all three components this is not a feasible option.

The findings from Chapter 3 further highlight that a GIP/xenin hybrid in combination with the GLP-1 analogue, exendin-4, has the capacity to restore the beneficial actions of GIP which are diminished in T2DM, as well as GLP-1 related actions. More importantly, this treatment paradigm has favourable significant persistent effects, which lasted 14-days after cessation of treatment, which is an exciting prospect given the progressive nature of T2DM. Thus, this hybrid may be useful as a new drug class for the treatment of T2DM.

7.6 Novel modified truncated forms of xenin in obesity-diabetes

The degradation fragments of native xenin, first discovered in 1992 [Feurle *et al*. 1992], have more recently been fully identified [Martin *et al*. 2014]. Therapeutically, xenin has been shown to exert dual beneficial properties, including independent anti-diabetic effects and GIP additive effects. However, only the fragment peptide, xenin-8, was shown to elicit the biological actions of the parent peptide [Martin *et al*. 2014, 2016]. Truncated peptides hold pharmacological advantages, including ease and cost of synthesis and enhanced efficacy. However, these truncated peptides are still prone to enzymatic degradation, with Lys and Arg regions within xenin known to be linked to enzymatic cleavage sites [Martin *et al*. 2014]. Thus, amino acid substitution of these Lys and Arg residues to Gln within the parent peptide and xenin-8 was shown to generate stable, potent peptides [Parthsarathy *et al*. 2016; Martin *et al*. 2016]. Studies within our laboratory have already characterised the potent actions of xenin-8-Gln [Martin *et al*. 2016], with initial characterisation and assessment of hybrid peptides incorporating xenin-8-Gln being performed [Hasib *et al*. 2018a, 2018b, 2019; Craig *et al*. 2019]. However, full characterisation of the biological activity of xenin-6 has been lacking until now.

In chapter 4, modified forms of xenin-6 were developed, using C-terminal amidation, amino acid substitutions or introduction of a reduced peptide bond to increase stability. However, the reduced pseudopeptide bond was the only modification employed within xenin-6 that elicited stability, which corresponds to previous research that the introduction of a reduced pseudopeptide bond produced an enzymatically stable peptide [Feurle *et al*. 2003]. Xenin has established dual therapeutic application, due to its insulinotropic and glucose-lowering actions, along with its ability to augment the diminished incretin effect by GIP in T2DM [Wice *et al*. 2010; Taylor *et al*. 2010; Martin *et al*. 2012, 2014; Parthsarathy *et al*. 2016; Craig *et al*. 2019]. These dual actions were also confirmed in truncated xenin-6 peptides. The insulinotropic capabilities were established in all modified hexapeptides assessed, in both BRIN-BD11 cells and isolated islets, but to a greater degree by Ψ-xenin-6. This demonstrated that these C-terminal hexapeptides are capable of triggering the insulin-secreting signalling pathways involved between xenin and pancreatic beta cells. However, further investigations are required to establish how exactly these insulinotropic effects of xenin are elicited, as many potential mechanisms have been suggested, including acting through neurotensin receptors [Clemens *et al*. 1997]. Although studies within our laboratory and others have demonstrated that these effects are likely to be independent of neurotensin receptors [Heuser *et al*. 2002; Taylor *et al*. 2010], and thus further assessments are required to clarify the mechanism/s of action. The hexapeptides assessed also exhibited GIP additive effects. In harmony with the parent peptide [Leckstrom *et al*. 2009], Ψ-xenin-6 was also shown to exhibit appetite suppressive actions. This confirms that the reduced pseudopeptide bond does not compromise peptide biological activity, but may improve bioactivity. In addition, glucose-lowering and insulinotropic activity of Ψ-xenin-6 was also retained up to 8

hours, superior over all other xenin hexapeptides examined, indicating enhanced duration of biological action through insertion of a pseudopeptide bond. Thus, chapter 4 highlights the favourable anti-diabetic and anti-obesity effects of novel hexapeptides of xenin, in particular the exciting potential of Ψ-xenin-6, and warrants further investigation to elucidate further therapeutic benefits in T2DM/ obesity models.

7.7 Augmenting the biological actions of sitagliptin through co-administration with xenin

Chapter 4 highlighted the exciting acute anti-diabetic potential of Ψ-xenin-6 *in vitro* and *in vivo.* Therefore, the rationale behind chapter 5 was to elucidate sub-chronic effects of this stable, long-acting peptide, and its ability to augment the established anti-diabetic benefits of the established DPP-4 inhibitor sitagliptin through co-administration in HFF mice. Ψ-xenin-6 alone did elicit various benefits in HFF mice, which is intriguing given the reduced dose employed compared to sitagliptin. These observations could be due to the independent insulinotropic benefits of xenin or through the augmentation of GIP by Ψ-xenin-6 as established in chapter 4. However, further studies are needed to confirm its mechanism of action and signalling pathways involved. Surprisingly, favourable effects on energy intake were lacking in both Ψ-xenin-6 alone and in the combination treated group, which is unexpected given the appetite suppressive effects shown by Ψ-xenin-6 in chapter 4, and by xenin in general [Alexiou *et al*. 1998; Cline *et al*. 2007; Cooke *et al*. 2009; Taylor *et al*. 2010]. However, favourable weight reductions were apparent, independent of feeding patterns, and therefore analysis of energy expenditure would have been helpful in understanding this effect. Following 18-day treatment, and consistent with acute effects noted in chapter 4, Ψ-xenin-6 elicited insulin secretory and glucose lowering

actions. Chapter 4 also demonstrated benefits of Ψ-xenin-6 on beta cells that was confirmed in this chronic study.

As expected, combination therapy did induce numerous metabolic benefits in HFF mice, and the benefits of sitagliptin were further augmented through combination therapy with this hexapeptide. To explore potential mechanism(s) of these beneficial effects a DPP-4 activity assay was conducted. As expected sitagliptin decreased DPP-4 activity of plasma following 18-days treatment in HFF mice. Importantly, this was not altered by concurrent Ψ-xenin-6 treatment, with these observations further corroborated using established *in vitro* methodologies. As such, Ψ-xenin-6 does not augment elevations of GIP and GLP-1 induced by sitagliptin, and does not enhance the DPP-4 inhibitory action of this drug. Thus, the benefits of the combined sitagliptin and Ψ-xenin-6 treatment regimen are most likely related to augmentation of GIP bioactivity by Ψ-xenin-6, as has been previously demonstrated. As anticipated, sitagliptin significantly increased levels of both incretin hormones, whether administered alone or in combination with Ψ-xenin-6. Interestingly, treatment with Ψ-xenin-6 alone increased plasma GIP levels, but this effect was not additive with sitagliptin, whereas high fat feeding decreased circulating GLP-1 concentrations.

Previous studies have omitted xenin based therapeutics as potential antidiabetics due to the suggestion that xenin may act to decrease GLP-1 concentrations [Sterl *et al*. 2016], however in this study no antagonistic effects on GLP-1 secretion was apparent. Also, given the favourable effects of combination therapy on gene expression in the liver, linked with reductions in weight gain, additional insight on lipid profiles would have been interesting to assess if insulin sensitivity in the liver was improved. To

further assess the effects of therapies examined on expression of genes, RNAseq on murine samples could be performed.

In conclusion, chapter 5 demonstrated the clear antidiabetic potential of Ψ-xenin-6 alone, and highlighted that a combinational treatment approach of Ψ-xenin-6 with sitagliptin can further augment the established therapeutic benefits of sitagliptin. This demonstrates that targeting both arms of the incretin axis provides clear antidiabetic and anti-obesity qualities and thus represents a suitable novel treatment option for obesity / diabetes.

7.8 Anti-obesity and anti-diabetic potential of combination therapy of an angiogenesis inhibitor and sitagliptin

Chapter 5 demonstrated that the favourable anti-diabetic benefits of sitagliptin can be further augmented through combination therapy. However, a drawback of sitagliptin monotherapy is its weight neutral effects. Therefore, in chapter 6 we employed the MetAP2 inhibitor TNP-470, which has been shown to exhibit potent weight-reducing effects, as well as anti-diabetic capabilities, in combination with the established therapeutic sitagliptin. A major advantage of employing combination therapy is the possibility of lower dose administration compared to either treatment alone [Irwin and Flatt, 2015]. This was apparent in chapter 6, as sub-maximal doses still exhibited significant potent beneficial effects, thus reducing patient drug-exposure and limiting off-target adverse effects as previously described for the use of GLP-1 based therapies [Butler *et al*. 2013b]. The anti-diabetic benefits shown by TNP-470 treatment, could be linked to xenin-related pathways, as a consequence of inhibiting xenin degradation. This subsequently resulted in the potentiation of GIP-associated metabolic benefits,

and improvement in diabetic status with augmented GIP concentrations. Thus, combination therapy of TNP-470 with sitagliptin targeted both incretin hormones and xenin signalling pathways, resulting in favourable insulinotropic and anti-hyperglycaemic effects, as well as substantial weight reducing effects by TNP-470. Further studies using mass spectrometry could be used to confirm the elevated concentrations of these hormones in plasma. This treatment paradigm also offers exciting anti-obesity therapeutic potential as shown through decreased energy expenditure, reduced food intake coupled with substantial weight-reducing properties. Indeed, assessment of body fat mass would have been interesting to consider in view of the roles GIP and xenin play in lipid metabolism [McIntosh *et al*. 2009; Bhavya *et al*. 2017]. Further analysis of adipocyte action using *in vitro* models and *ex vivo,* imaging would be insightful, along with studies examining the effects of xenin on adipogenic-specific genes or proteins.

Oral delivery is a big patient benefit over peptide therapies. In this study, i.p. injection was utilised to allow for reduced doses, ensure consistent dosing of animals, and negate need for specialised vehicle. Essentially, this study was a 'proof-of-concept' study, which has confirmed that the concept works, thus the next step would be to perfect oral delivery methods and doses.

Therapies which target both obesity and diabetes would offer superior metabolic benefits for T2DM [Skow *et al*. 2016], due to the considerable links between obesity and diabetes. This chapter confirmed the importance of concurrently targeting both incretin hormones within the incretin axis, along with targeting mechanisms involved in exhibiting anti-obesity effects. Co-administration of sitagliptin with TNP-470

displayed both anti-diabetic and anti-obesity effects, which is highly promising considering the parallel escalations in obesity and T2DM, and thus should be explored as a novel treatment paradigm. As evident from this thesis, the concept of multi-functional peptide targeting combinations is exciting and requires further progression towards the clinic.

7.9 Future studies

This thesis has highlighted the potential of modified truncated forms of xenin and subsequent elevations in GIP concentrations in alleviation of diabetes and obesity, particular through improvement of metabolic control and additive GIP actions. Combination therapy with established drug classes also possess vast advantages over monotherapy, and allows lower dose administration, which is highly favourable moving towards the clinic. Nevertheless, future studies are required to fully elucidate the signalling pathways and mechanisms involved in the satiety effects, insulinotropic, and GIP-potentiating actions demonstrated by xenin to fully understand the therapeutic potential of the therapies investigated. Future studies should aim to identify a specific xenin receptor, and clarify the involvement, if any, of neurotensin receptors. Indeed, this could involve incubation of test peptides with various receptor transfected cell lines, for example, a neurotensin-receptor 1 transfected cell line and measurement of insulin secretion. Other mechanistic studies that could be employed include the use of antagonists, blocking key pathways or receptors, to help enlighten which receptors or pathways are involved in these beneficial effects. Similarly, specific receptors in beta cells could be knocked out using CRISPR/Cas 9 technology to establish essential receptors for these anti-diabetic effects. The GIP potentiating effects of xenin could also be examined in GIP-receptor knockout mice, to see if the

potentiating effects are still present. Finally, following on from the encouraging results from this thesis, the next step should be recruitment of Ψ-xenin-6 into clinical trials, given its superior stability, strong insulinotropic effects and prominent enhancements on metabolic profile both alone and in combination with established anti-diabetic therapies. Otherwise, Ψ-xenin-6 could also be incorporated into a hybrid peptide agent, as hybrid agents offer a more patient friendly approach through one single injection of a multi-targeting peptide rather than multiple administration of mono-peptides. However, challenges are apparent with translation from animal model to human due to risk of off-target effects caused by cross-reactivity with other off-target receptors [Skow *et al*. 2016]. Accordingly, more than one animal model of diabetes/obesity should be used due to diversity seen in the human disease, along with further receptor affinity studies before conversion to a human setting.

Given the prevalence of both obesity and T2DM, current anti-diabetic therapies fail to successfully target the established links between both metabolic diseases. Therefore, it would be highly favourable, and a necessity, for a novel treatment to be established that could potentially act as an obesity preventative measure or a more potent management therapy, along with having antidiabetic efficacy. As such, further research into TNP-470 as a preventative measure or as a therapy is warranted in various obesity / diabetic mouse models, before progression to the clinic, either alone or in combination with reputable T2DM therapies.

7.10 Concluding remarks

To summarise, collectively the studies in this thesis demonstrate that truncated forms of xenin-25 retain the biological actions of the parent compound, and that elevated xenin concentrations exert beneficial metabolic effects through potentiation of GIP. Remarkably, (DAla^2)GIP/xenin-8-Gln in combination with exendin-4 retained persistent beneficial effects following cessation of treatment. Notably, the established benefits of sitagliptin can be further enhanced through combination therapy with xenin-related peptides, and that TNP-470 in combination with sitagliptin possess beneficial anti-hyperglycaemic and anti-obesity effects. This thesis endorses that monotherapy in T2DM is no longer an effective treatment option for this multifactorial disease, and that combination therapy with either gut hormones or hybrid peptides has potential to emerge as a leading therapeutic approach for T2DM. Taken together, the promising therapeutic actions displayed within this thesis will hopefully advance the therapeutic repertoire and result in the development of a safe, efficient, long-acting and cost-effective treatment option for obesity/T2DM.

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